Antibacterial resistance: A comparative study between clinical and environmental isolates.

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

Submitted by

Monisha Anindita

Student ID: 10226001

April 2015

Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University

DECLARATION

I hereby declare that the thesis project titled **"Antibacterial resistance: A comparative study between clinical and environmental isolates"** is my own work and the research work presented here is based on the actual and original work carried out by me. All the research work has been carried out under the supervision of Fahareen-Binta-Mosharraf, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, 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.

(Monisha Anindita)

Candidate

Certified

(Fahareen-Binta-Mosharraf)

Supervisor

Lecturer,

Microbiology Program,

Department of Mathematics and Natural Sciences,

BRAC University, Dhaka.

Acknowledgement

The accomplishment of my research work in pursuance of my dissertation is the very first time that I have had the exposure to this kind of experimental research work. Completion of this work needed lots of help and encouragement so as not to get frustrated at any event of failure or whenever I stumbled upon any difficulties during this elaborate time of the experimental works. I was fortunate enough to have the people around me who have provided me with all the needed support.

My regards, appreciation, indebtedness and deepest gratitude towards my respected supervisor **Fahareen-Binta-Mosharraf**, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, for her efforts and patience with me throughout the period of my research work. I am extremely fortunate to have her as my supervisor for she has constantly supervised me and bestowed upon me her constructive criticism, expert guidance and has given me moral support and help whenever my steps faltered during the whole period of my research work.

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 excellent guidance, monitoring and constant encouragement throughout the Project. His ever so welcoming nature and the enthusiastic will to help the students has helped me not only during this research work, but throughout my entire time in BRAC University.

I would also like to thank and express my deepest gratitude to **Dr. Mahboob Hossain**, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University who helped and guided me in my thesis report writing and has provided suggestions regarding the setting and execution of the experimental works, interpretation of the results and ensuing directions for the whole work with great patience. It would have been impossible to submit my thesis report without his cordial help.

Also, my appreciation goes to all my departmental teachers who have often enquired about the progress of my work and very often provided me with their valuable advice. I thank them all for this kind and affectionate gesture.

Finally, I extend my special thanks and gratitude to all my seniors and batch mates in the microbiology specialized research laboratory, who have provided good working environment by sharing their advice and encouragement to make me feel at ease even in the hardest times. They have willingly helped me out with their valuable information and boosted my efforts in finishing the task properly. Without their cordial support it would be very difficult to carry out this research work.

Monisha Anindita

April, 2015

Abstract

This comparative study was carried out to investigate the presence of antibiotic sensitive and resistant bacteria in different environmental samples of Bangladesh with specific clinical strain obtained from ICDDR, B .The environmental samples were isolated from the month April 2014 to September 2014 from different locations of Dhaka city to perceive the propensity of the environmental isolates to develop antibiotic resistance at different time periods of a year. The bacterial isolates were identified on the basis of standard cultural, morphological and biochemical characteristics. Antibiogram was done to identify the antibiotic susceptibility of the isolates according to CLSI guideline. The result of this study indicated that significant number of the environmental isolates has become resistant to the tested antibiotics, and some of them have become multi-resistant to these therapeutic agents whereas all the clinical isolates showed the opposite result. The study helps to predict the future emergence and guide the development of strategies to counteract this resistance. Therefore periodic and comprehensive survey of antibiotic resistance in the environmental bacteria is required.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

❖ BPA	Baird-Parker Agar
❖ CFU	Colony Forming Unit
❖ EMB	Eosin Methylene Blue Agar
❖ MAC	MacConkey Agar
❖ ICDDR,B	International Center for Diarrheal Disease Research, Bangladesh
❖ IMViC	Indole, Methyl red, Voges-Proskauer, Citrate
❖ LB	Luria Bertani
❖ MSA	Mannitol Salt Agar
❖ MR	Methyl Red
❖ MRSA	Methicillin-resistant *Staphylococcus aureus*
❖ TSB	Trypticase Soy Broth
❖ MYP	Mannitol Egg Yolk Polymyxin Agar
❖ **NA**	**Nutrient Agar**
❖ TSI	Triple Sugar Iron
❖ XLD	Xylose Lysine Deoxycholate
❖ UTIs	Urinary Tract Infections
❖ SXT 25	Sulfamethoxazole / Trimethoprim
❖ FOX 30	Cefoxitin
❖ PEF 5	Pefloxacin
❖ CIP 5	Ciprofloxacin
❖ E 15	Erythromycin
❖ CN 10	Gentamicin
❖ K 30	Kanamycin
❖ S 10	Streptomycin

- CXM 30 Cefuroxime Sodium
- NA 30 Nalidixic Acid
- OX 1 Oxacillin
- C 30 Chloramphenicol
- F 300 Nitrofurantoin

Chapter 1: Introduction

Chapter 1: Introduction

1.1 Overview

It has long been established that, natural selection shapes the evolution of DNA based organisms, through the course of survival or extinction within the dynamic environment of the planet. Through breakthrough scientific discoveries, the all-connecting symbiotic nature of all living things have become more and more evident. With this knowledge it is now understood far better than ever that as humans, with a huge sustained footprint on the environment, we have become a substantial influence in the context of evolution. This idea is a bit hard to grasp as most of the noticeable evolution takes place over thousands to millions of years, but when it comes to microorganisms, it is fairly observable within human lifespan that these single celled creatures are adapting to the direct or indirect interactions with us and the environment as a whole, with ferocious rapidity.

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. These studies are carried out by various pharmaceutical companies, organizations for disease control, genetic engineers, biotechnologists, evolutionary microbiologists and every other group that have a vested interest in improving and maintain the quality of human life.

A stark example can be the evolution of the enteric disease causing pathogens throughout the history of modern medicine. 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 Emerging pathogens

A humongous number of organisms are associated with a variety of infectious diseases, and are considered to be human pathogens. Not only are the pre existing pathogens menacing the human health, but also some pathogens have only been recognized very recently. Such pathogens are largely accountable for a massive number of deaths every year. As many new diseases are held responsible for this global burden, which is taking its toll on human health, it is also true for diseases that had been declining over the past few years [1]. The re emergence of these diseases is associated with certain transmission routes and also different and wide range of hosts.

1.3 Bacterial resistance to antibiotics; now and then

Antibiotics, since its invention have been vital in the fight against infectious diseases that are caused by various bacteria as well as other microbes. A matter of great misery is that although we have all these advanced antibiotics now at our disposal, reports have shown that no antibiotic can actually last effective for too long. As a result, resistance in the pathogens to common therapeutic agents is increasing in recent years. Thus, a mere cultural contaminant or a harmless microbe can eventually become great human pathogen over time [2].

The abundance and indiscriminate use of the commercial antimicrobial drugs that are commonly used in the treatment of infectious diseases is the leading reason of multiple drug resistance in human pathogenic microorganisms that has been developed over the past years (Table 1) [5]. This development of antibiotic resistance has a number of favoring factors, those including the characteristics of the hosts, the usage of antibacterial agents, the specific nature of the relationship of bacteria to antibiotics, as well as environmental factors [3]. Since the bacterial pathogens have the ability to rapidly evolve, this helps them to come up with new ways to dodge the host defenses and become resistant to the antibiotic treatments. It is a matter of great concern that more and more pathogens are showing resistance to multiple drugs. In addition the high levels of antibiotics used in humans and animals now a day have amplified the emergence of these antibiotic resistant strains that are the main concern of this study [4].

Table 1: Profile of clinical pathogens used in this study

Test organisms	Infections	Antibiotic susceptibility
Bacillus cereus	Causes food-borne illness and chronic skin infection, also causes ocular infections	Vancomycin, Gentamycin, Chloramphenicol and Erythromycin
Salmonella typhi	Causes food poisoning and typhoid	Azithromycin, Ciprofloxacin
Shigella flexneri	Causes dysentery	Ampicillin, Ciprofloxacin, Tetracycline
Staphylococcus aureus	Causes skin and tissue infections	Vancomycin
E.coli K12	Causes urinary tract and wound infections, also problems after surgery	Cephaloridine

1.4 Aims and objectives

Resistance to antimicrobial agents has resulted in morbidity and mortality from treatment failures and increased health care costs. Even though defining the precise public health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that emergent antibiotic resistance is a serious global problem. Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance.

Although pharmaceutical industries have produced a number of new antibiotics in the last few decades but simultaneously the resistance of the microorganisms to these drugs have also increased. Due to such increasing resistance in microbes and synthetic antibiotic side effects, it is now necessary to access resistance pattern of pathogenic organisms against standard antibiotics to develop therapeutic alternatives.

On the basis of above context, the objectives of the present study are:

1. The degree of antibacterial activity will be evaluated considering the diameter (mm) obtained for the zone of inhibition on each of the replicate agar plate.

2. The resistance pattern of both clinical and environmental samples will be recorded for a comparative analysis.

Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

2.1 Working laboratory

All project works were performed in the Microbiology Specialized Research Laboratory, Department of Mathematics & Natural Sciences, BRAC University.

2.2 Reference Bacterial Strains

In this study, five standard clinical strain of *Bacillus cereus, E. coli K12, Salmonella typhi, Staphylococcus aureus,* and *Shigella flexneri* were used. All these species were obtained from ICDDR, B (International Center for Diarrheal Disease Research, Bangladesh).

2.2.1 Preparation of plating bacteria

- A single bacterial colony of each clinical isolates was inoculated into a screw cap test tube containing 5 ml Luria Bertani (LB) broth.
- The tube was incubated at 37^oC in a shaking incubator (Daihan Scientific, Korea) at 120 rotations per min (rpm) for 3 hours.
- After incubation 1 ml culture from the test tube was taken in an eppendorf and centrifuged (Eppendorf, Germany) at 12,000 rpm for 10 min and the supernatant was discarded.
- Bacterial pellet was suspended in 1 ml of 10 mM $MgSO_4$ properly to make it homogenous.
- The suspension was stored at 4^oC until used.
- The suspensions were then diluted 1:100 in TSB broth to obtain 10^6 CFU/ml before use.

2.2.2 Confirmation of the reference strains

Reference bacterial strains were identified routinely to distinguish each organism by sub culturing on recommended selective media (Table 2). The cultural properties of each organism were observed and recorded.

Table 2: Respective Selective Media for Different Reference Strains

Bacterial Strain	Selective Media
E .coli K12	MacConkey agar and Eosin-Methylene blue (EMB) agar
Salmonella typhi	Xylose Lysine Deoxycholate agar (XLD) agar
Bacillus cereus	Mannitol-Egg Yolk-Polymyxin Agar (MYP)
Shigella flexneri	Xylose Lysine Deoxycholate (XLD) agar
Staphylococcus aureus	Mannitol Salt agar (MSA)

2.2.3 Biochemical confirmation of the clinical strains

Subsequently each bacterial clinical strain were employed for morphological and biochemical confirmation tests. Recommended biochemical tests like Methyl red test, Voges–Proskauer test, Indole test, Citrate utilization test, Oxidase test, Catalase test, TSI agar test were performed for each of the strains.

2.2.4 Biochemical Identification

Biochemical tests were performed with specific standard isolates developed in specific media according to the standard methods described in Microbiology Laboratory Manual [6]. Before starting the process of any biochemical identification test, all the bacterial cultures were grown on nutrient agar plates in the incubator at 37^{o}C.

Methyl red (MR) test

- Bacterium to be tested was inoculated into 3 ml dextrose phosphate broth (MR-VP broth), which contained dextrose and a phosphate buffer and incubated at 37^{o}C for 24 hours.
- Over the 24 hours, the mixed-acid producing organism might produce sufficient acid to overcome the phosphate buffer and remain acidic.
- The pH of the medium was tested by the addition of five drops of MR reagent. Development of a red color was taken as a positive result. MR negative organism would give a yellow color.

Voges–Proskauer test

- Bacterium to be tested was inoculated into 3 ml dextrose phosphate broth (MR-VP broth) and incubated at 37^{o}C for 24 hours.
- To the aliquots of each broth cultures 10 drops of Barritt's reagent A was added and the cultures were shaken.
- Immediately, 10 drops of Barritt's reagent B was added and the cultures were shaken again.
- Cultures were then kept aside for 15 minutes for the reaction to occur.
- 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.

Indole test

- Bacterium to be tested was inoculated in 6 ml peptone water, which contains the amino acid tryptophan and incubated at 37^{o}C for 24 hours.
- Following overnight incubation, five drops of Kovac's reagent were added.
- 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.

Citrate utilization test

- A single bacterial colony of each bacterium to be tested was picked up from each nutrient agar plates by a needle and inoculated into the slope of Simmon's citrate agar and incubated at 37^{o}C for 24 hours.
- Over these 24 hours, the organism which had the ability to utilize citrate would change the color of the media.
- All the media that changed their color from green to a Prussian blue, is taken as a positive result. A negative slant would have no growth of bacteria and would remain green.

Triple Sugar Iron (TSI) Test

- A single bacterial colony of each bacterium to be tested was picked up from each nutrient agar plates by a needle and stabbed into the TSI containing dextrose, lactose and sucrose butt.
- Caps of the tubes were loosened and incubated at 35°C for overnight and were examined after 18-24 hours for carbohydrate fermentation, CO_2 and H_2S production.
- A yellow (acidic) color in the butt indicated that the organism being tested is capable of fermenting all the three sugars, whereas a red (alkaline) color in the slant and butt indicated that the organism being tested is a non fermenter.
- A black precipitation in the butt of the tube is the indication of H_2S production.
- Presence of bubbles, splitting and cracking of the medium is the indication of CO_2 gas production.

Oxidase test

- Two drops of oxidase reagent (*p*- Aminodimethylaniline oxalate) were added onto the filter paper (Whatman, 1MM).
- A loopful of each bacterium to be tested 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.

Catalase test

- One drop of catalase test reagent (hydrogen peroxide) was placed on a sterile glass slide.
- An isolate from a nutrient agar plate was picked up with a sterile toothpick and placed on to the reagent drop.
- This was done with each of the bacterium to be tested.
- An immediate bubble formation indicates a positive result.

2.2.5 Preparation of Stock Sample

Short term preservation

3 ml of T_1N_1 agar butt in each vial was inoculated by stabbing isolates from nutrient agar plates. Then the vials were incubated at 37^{o}C (SAARC) for 6 hours. After incubation, the surface of the medium was covered with 200 µl sterile paraffin oil and the vials were stored at room temperature appropriately labeled.

Long term preservation

For long-term preservation, 500 µl of bacterial culture grown in Trypticase Soy Broth at 37^{o}C for 6 hours was taken in a sterile cryovial. Then 500 µl of sterile glycerol was added to the broth culture and the cryovial was stored at -20^{o}C.

2.3 Provided Antibiotic Discs

In this study, the effectiveness of thirteen different antibiotics was determined. They are listed below in the table:

Table 3: Provided Antibiotic Discs

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

2.4 Antibiotic Susceptibility Test of the Standard Clinical Strains of Bacteria

The standard clinical strains of bacteria were tested for their sensitivity against thirteen standard antibiotics, as mentioned previously in Table 3.

2.4.1 Preparation of McFarland Solution

McFarland solution is an essential material needed before testing the microorganisms for their sensitivity. McFarland standards are used as reference to adjust the turbidity of any given bacterial suspension. This is done to make sure that the number of bacteria is within a given range to standardize the microbial testing. This would also help avoid any error in result, because if the suspension is too heavy or too diluted, an erroneous result might occur for any given anti microbial agent, which in this study, is antibiotic discs.

- 97% H_2SO_4 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 $BaCl_2.2H_2O$.

2.4.2 Preparation of inoculums

- Using a sterile inoculating loop, one or two colonies of the organism to be tested were taken from the subculture plate.
- The organism was suspended in 6 ml of physiological saline.
- The test tube containing the saline was then vortexed to create an overall smooth suspension.

2.4.3 Comparing with McFarland solution

- Using the Colorimeter (Labtronics; ISO 9001: 2008 Certified), the OD of the McFarland solution was measured to be 11.
- Then the OD of each of the inoculums that were made was also measured with the Colorimeter.
- Only the solutions with the OD that matched with that of the McFarland solution were taken.
- The solutions that gave an OD higher than the standard solution were diluted with solution to match the standard.
- Once all the OD of the inoculums was matched with the standard, they were ready to be inoculated on MHA (Muller-Hinton Agar) plates.

2.4.4 Inoculation of the MHA plates

- A sterile swab was dipped into the inoculum tube. The swab was rotated against the side of the tube above the fluid level, using firm pressure, to remove excess fluid, but the swab was not dripped wet.
- The dried surface of a MHA plate was inoculated by streaking the swab four to six times over the entire agar surface; the plate was rotated approximately 60 degrees each time to ensure an even distribution of the inoculums.
- The plate was rimmed with the swab to pick up any excess liquid.
- Leaving the lid slightly ajar, the plate was allowed to sit at room temperature at least 3 to 5 minutes for the surface of the agar plate to dry before proceeding to the next step.

2.4.5 Placement of the Antibiotic Discs

- The forceps was sterilized by immersing the forceps in alcohol then igniting.
- Four sterile discs were placed on the surface of an agar plate, using that forceps.
- The discs were gently pressed with the forceps to ensure complete contact with the agar surface.
- Placing 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.
- Once all discs were in place, the plates were inverted, and placed in a 37°C incubator for 24 hours.

2.4.6 Measuring zone size

- Following the incubation, the zone sizes were measured to the nearest millimeter using a ruler.
- All measurements were made with the unaided eye while viewing the back of the Petri dish.
- The zone size was recorded on the recording sheet.

2.5 Environmental sample

Different types of environmental samples were collected from different sources in the month of October, 2014 (Table 4). Throughout the month of October, the daytime temperatures had generally reached highs of around 30°C, which is about 86°F. At night, the average minimum temperature dropped down to around 21°C, which is 70°F. The average daily relative humidity for October was around 84%.

Table 4: Source of environmental samples

Environmental Sample	Collected from
Chicken feces	a chicken farm located at Banani
Cow dung	a farm located at Gulshan
Handmade salad	a restaurant located at Mohakhali
Human sewage	The river Buriganga

2.5.1. Sample processing

Six fold serial dilutions were done with the different environmental samples following the standard sample processing technique.

- 1 ml of sample suspension was taken and mixed with 9 ml of physiological saline in a test tube and the tube was vortexed properly to make a smooth and even suspension.
- Then the sample was diluted six times to get the desired concentration.
- The same procedures were followed for all four of the samples.

2.5.2. Assortment of desired colonies from nutrient agar plates

The maximum diluted sample was spread on nutrient agar plate for total count. Multiple colonies from each of the nutrient agar plates were selected depending on the cultural characteristics and transferred to specific standard selective media for further confirmation.

2.5.3 Confirmation of the organisms by biochemical tests

Multiple selected presumptive colonies from the specific media were confirmed by biochemical tests following standard protocol [6], as was previously done with the reference strains.

2.5.4 Antibiotic susceptibility test of the microorganisms from environmental samples

All the steps and methods that were followed during the antibiotic susceptibility test of the reference bacterial strains were repeated with the microorganisms now isolated from the environmental samples.

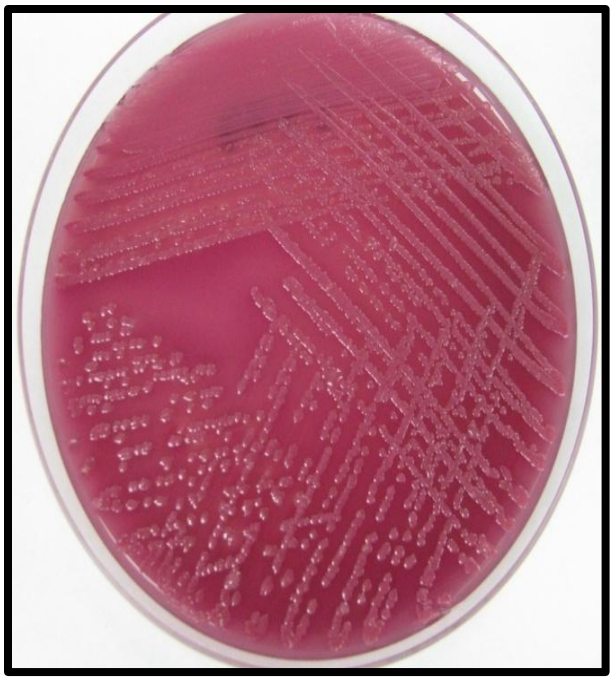
Chapter 3: Results

3.1 Conformation of clinical strains

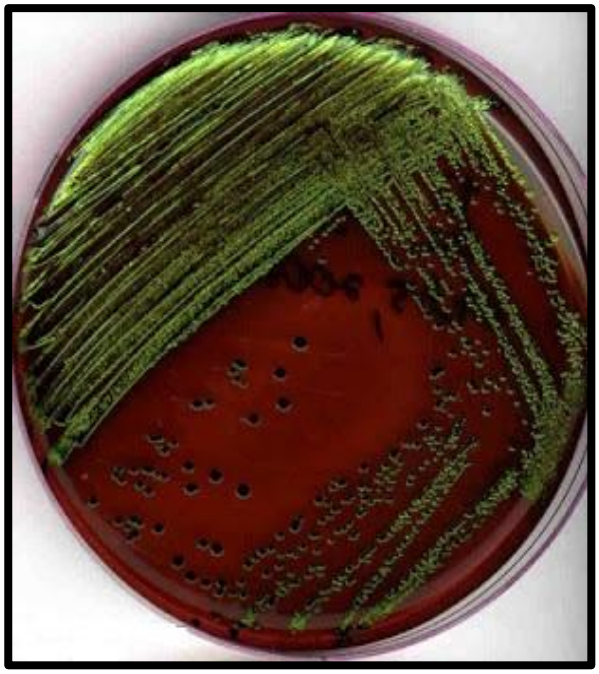
Clinical strain of the five bacterial species i.e. *Escherichia coli* strain K12, *Bacillus cereus*, *Salmonella typhi*, *Shigella flexneri*, *Staphylococcus aureus* obtained from ICDDR,B (International Center for Diarrheal Disease Research, Bangladesh) were preliminary identified by their cultural properties (Table 5) upon streaking in the respective selective media (Table 2). Selective medium types are formulated to support the growth of one group of organisms, but inhibit the growth of another. These media contain antimicrobials, dyes, or alcohol to inhibit the growth of the organisms that are not targeted for study.

Table 5: Cultural characteristics of clinical strains on respective selective media

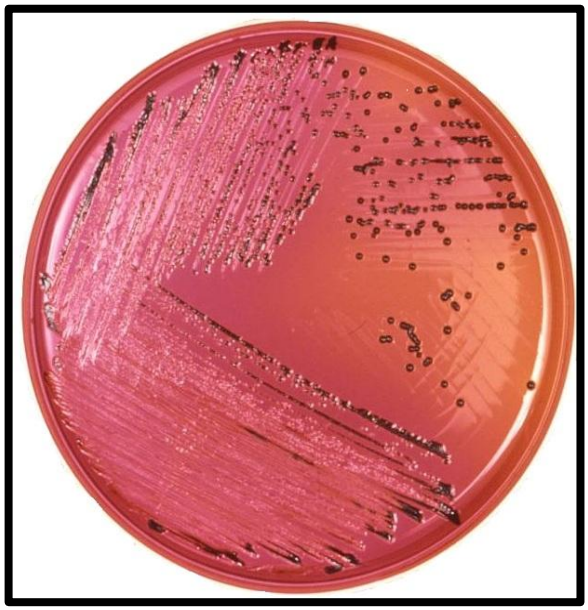
Isolates/ Organism	Cultural Characteristics						
	Medium	**Size**	**Margin**	**Elevation**	**Form**	**Pigment**	**Consistency**
B. cereus	MYP agar	Large (4-5mm)	Undulate	Raised	Circular	Bright pink colonies with egg yolk precipitation	Creamy, smooth
S. typhi	XLD	Moderate (2-3mm)	Entire	Raised	Convex	Red colonies with black centre	Smooth
S. flexneri	XLD	Moderate (1-2mm)	Entire	Convex	Circular	Pinkish to reddish colonies	Smooth
S. aureus	MSA	Moderate (2-3mm)	Entire	Convex	Circular	Yellow	Smooth
E. coli K12	MAC	Large (2-3mm)	Entire	Raised	Circular	Pink	Smooth
	EMB	Large (2-3mm)	Entire	Slightly raised	Circular	Blue-black colonies with metallic green sheen	Shiny, smooth



(a)

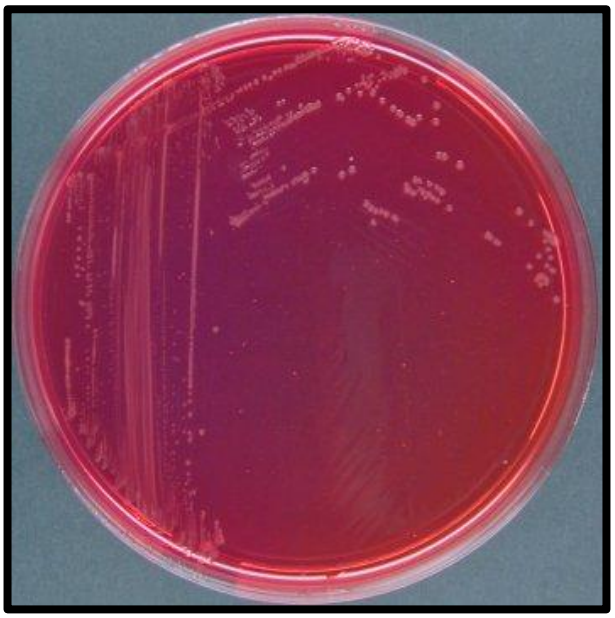


(b)



(c)

(d)



(e) (f)

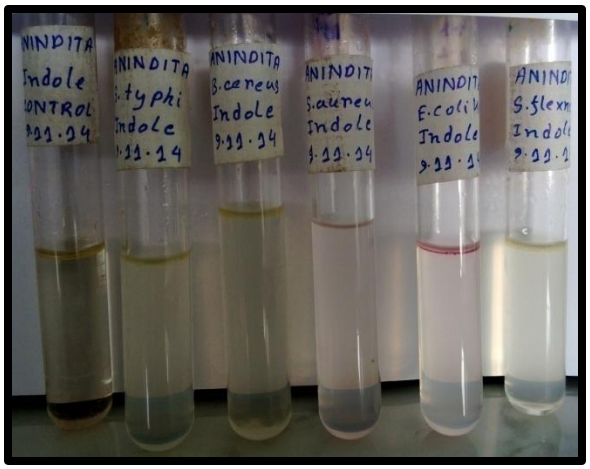
Figure 1: Cultural characteristics of clinical strains on respective selective media:
(a) *Escherichia coli* K12 in MacConkey agar,
(b) *Escherichia coli* K12 in Eosin Methylene blue agar,
(c) *Salmonella typhi* in Xylose Lysine Deoxycholate agar,
(d) *Bacillus cereus* in MYP agar,
(e) *Staphylococcus aureus* in Mannitol Salt agar,
(f) *Shigella flexneri in* Xylose Lysine Deoxycholate agar

Isolates shown in Figure 1 showed patterns of biochemical reactions that are typical for each strain of bacteria according to Microbiology Laboratory Manual [6]. In Table 6 below, the results of biochemical tests of the clinical isolates are mentioned.

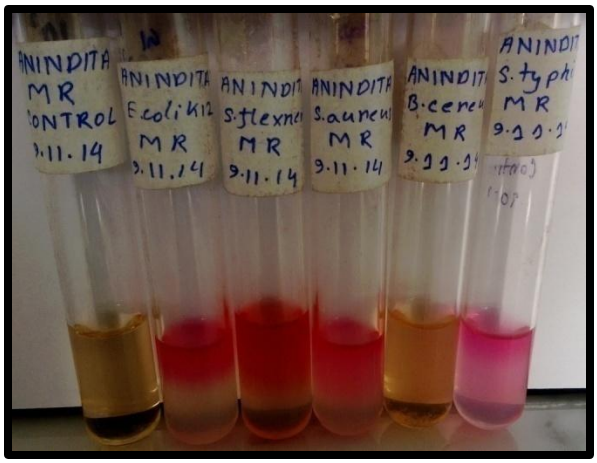
Table 6: Standard results of biochemical tests of target strains

Isolate/Organism	Biochemical Tests									
	Indole production test	Methyl red reaction test	Voges Proskauer reaction test	Citrate utilization test	TSI fermentation				Catalase activity test	Oxidase activity test
					Slant	Butt	CO_2	H_2S		
Bacillus cereus	-	-	-	-	**A**	**A**	-	-	+	+
Salmonella typhi	-	+	-	-	**K**	**A**	-	+	+	-
Shigella flexneri	-	+	-	-	**K**	**A**	-	-	+	-
Staphylococcus aureus	-	+	-	-	**A**	**A**	+	-	+	-
E. coli K12	+	-	-	+	K	K	+	-	-	-

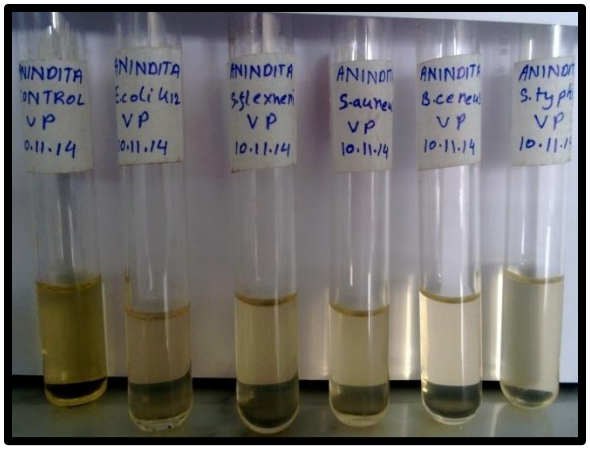
KEY: A= acidic condition, K= alkaline condition, + = positive, - = negative, AG= both acid & gas production.



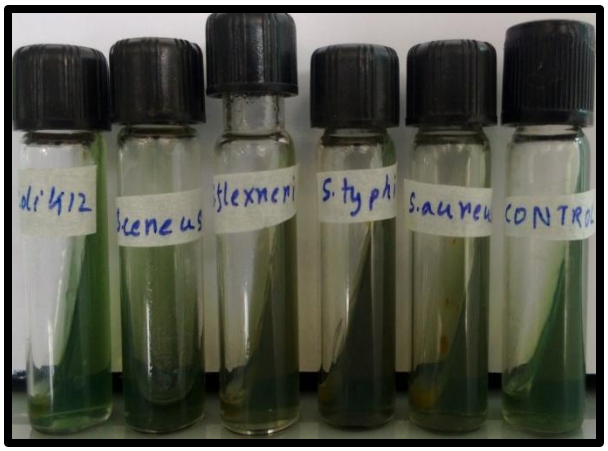
(a)



(b)



(c)



(d)

Figure 2: IMViC test results; (a) Indole test, (b) Methyl red test, (c) Voges Proskauer test, (d) Citrate utilization test

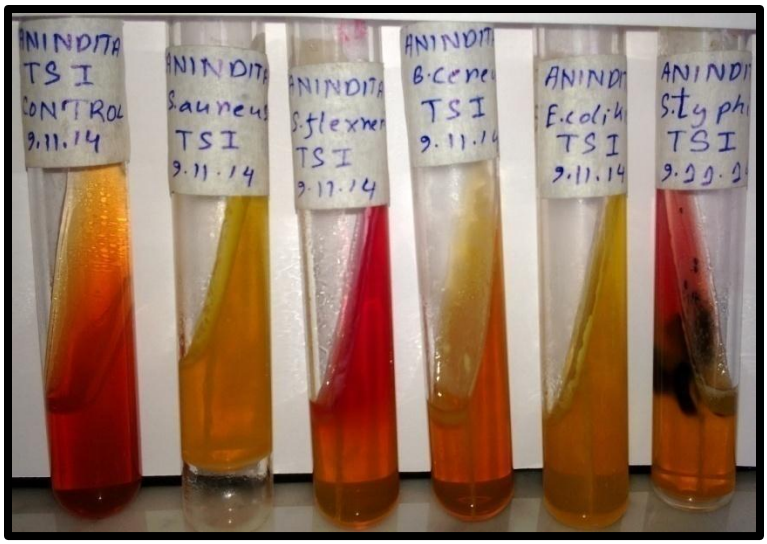


Figure 3: Triple Sugar iron (TSI) test

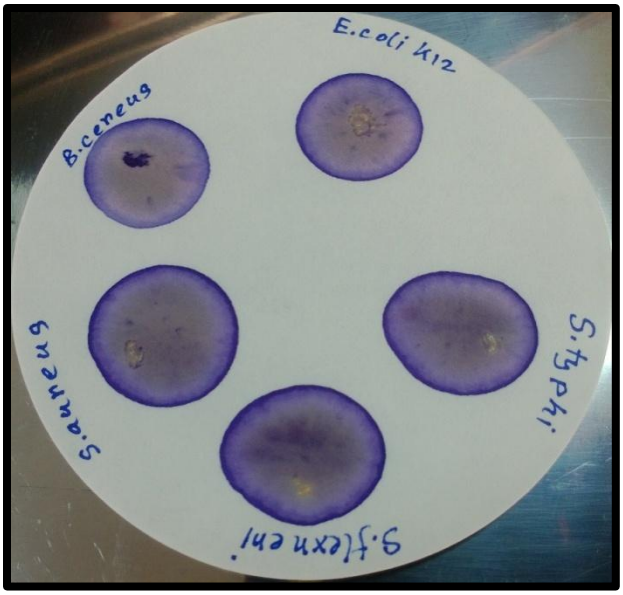


Figure 4: Oxidase Test

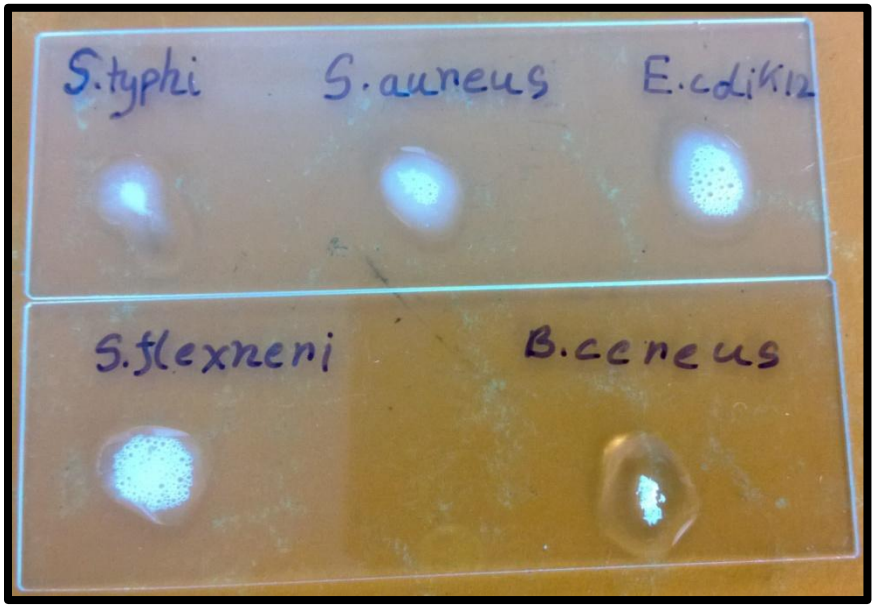


Figure 5: Catalase test

3.2 Confirmation of the environmental strains by biochemical tests

Desired presumptive environmental strains of *Escherichia coli* strain K12, *Bacillus cereus*, *Salmonella typhi*, *Shigella flexneri*, and *Staphylococcus aureus* are selected from specific media (Table 2) depending on their cultural characteristics (Table 7).

Table 7: Cultural characteristics of the environmental strains

Isolates/ Organisms	Cultural Characteristics						
	Medium	**Size**	**Margin**	**Elevation**	**Form**	**Pigment**	**Consistency**
Bac 1	MYP agar	Large (4-5mm)	Undulate	Raised	Circular	Bright pink colonies with egg yolk precipitation	Creamy, smooth
Bac 2	MYP agar	Moderate (1-2mm)	Entire	Convex	Irregular	Colorless	Smooth
Bac 3	MYP agar	Moderate (2-3mm)	Entire	Flat	Convex	Pinkish	Smooth
Bac 4	MYP agar	Large (2-3mm)	Entire	Slightly Raised	Irregular	Yellowish	Smooth
Sal 1	XLD	Moderate (2-3mm)	Entire	Convex	Circular	Colorless	Creamy
Sal 2	XLD	Small (1-2mm)	Entire	Convex	Circular	Pinkish	Smooth
Sal 3	XLD	Moderate (2-	Entire	Raised	Convex	Red colonies	Smooth

		3mm)				with black centre	
Sal 4	XLD	Large (2-3mm)	Undulate	Slightly raised	Irregular	Colorless	Smooth
Shi 1	XLD	Moderate (2-3mm)	Entire	Raised	Convex	Pink	Smooth
Shi 2	XLD	Large (2-3mm)	Entire	Raised	Irregular	Colorless	Smooth
Shi 3	XLD	Moderate (1-2mm)	Entire	Convex	Circular	Pinkish to reddish colonies	Smooth
Shi 4	XLD	Small (1-2mm)	Entire	Slightly raised	Circular	Colorless	Creamy
Sta 1	MSA	Moderate (2-3mm)	Entire	Raised	Circular	Yellowish	Smooth
Sta 2	MSA	Moderate (2-3mm)	Entire	Convex	Circular	Yellow	Smooth
Sta 3	MSA	Large (2-3mm)	Entire	Slightly raised	Ireregular	Colorless	Creamy
Sta 4	MSA	Small (1-2mm)	Entire	Convex	Circular	Colorless	Smooth

Esc 1	MAC	Small (1-2mm)	Entire	Flat	Circular	Pink	Rough
	EMB	Moderate (2-3mm)	Entire	Flat	Circular	Pink	Smooth
Esc 2	MAC	Small (1-2mm)	Entire	Raised	Circular	Purple	Smooth
	EMB	Small (1-2mm)	Entire	Raised	Circular	Pink	Smooth
Esc 3	MAC	Small (1-2mm)	Entire	Slightly raised	Circular	Slight Pink	Rough
	EMB	Moderate (2-3mm)	Entire	Flat	Circular	Colorless	Smooth
Esc 4	MAC	Large (2-3mm)	Entire	Raised	Circular	Pink	Smooth
	EMB	Large (2-3mm)	Entire	Slightly raised	Circular	Blue-black colonies with metallic green sheen	Shiny, smooth

A total of twenty colonies belonging to five target strains isolated from different environmental sources (Table 4) were selected depending on the presumptive cultural characteristics which were further confirmed by standard biochemical tests showed below in Table 8.

Table 8: Biochemical test results of the environmental strains

Isolate/Organism	**Biochemical Tests**									
	Indole production test	**Methyl red reaction test**	**Voges Proskauer reaction test**	**Citrate utilization test**	**TSI fermentation**				**Catalase activity test**	**Oxidase activity test**
					Slant	**Butt**	**CO_2**	**H_2S**		
Bac 1	-	-	-	-	**A**	**A**	-	-	+	+
Bac 2	-	_	-	+	K	K	+	-	-	-
Bac 3	+	-	-	-	K	K	-	+	-	+

Bac 4	**+**	**+**	**-**	**-**	A	K	+	-	+	-
Sal 1	-	+	-	-	A	A	+	-	+	+
Sal 2	-	-	+	-	K	A	+	-	+	-
Sal 3	**-**	**+**	**-**	**-**	**K**	**A**	**-**	**+**	**+**	**-**
Sal 4	+	+	-	-	A	K	-	-	-	+
Shi 1	+	-	+	+	K	K	+	-	-	-
Shi 2	-	-	+	+	K	A	-	+	+	-
Shi 3	**-**	**+**	**-**	**-**	**K**	**A**	**-**	**-**	**+**	**-**
Shi 4	+	-	-	-	K	A	-	+	-	+
Sta 1	+	-	+	-	A	K	+	+	+	+
Sta 2	**-**	**+**	**-**	**-**	**A**	**A**	**+**	**-**	**+**	**-**

Sta 3	-	-	+	-	A	K	+	-	+	-
Sta 4	-	-	+	-	K	K	-	-	+	-
Esc 1	+	-	-	+	K	K	+	-	-	-
Esc 2	-	+	-	-	K	A	-	-	+	-
Esc 3	-	-	-	+	K	A	+	-	+	-
Esc 4	+	-	-	+	K	K	+	-	-	-

According to Table 7 and 8, Bac 1, Sal 3, Shi 3, Sta 2, and Esc 4 showed standard cultural characteristics as well as standard biochemical test results following Table 5 and 6, hence they were selected for further studies.

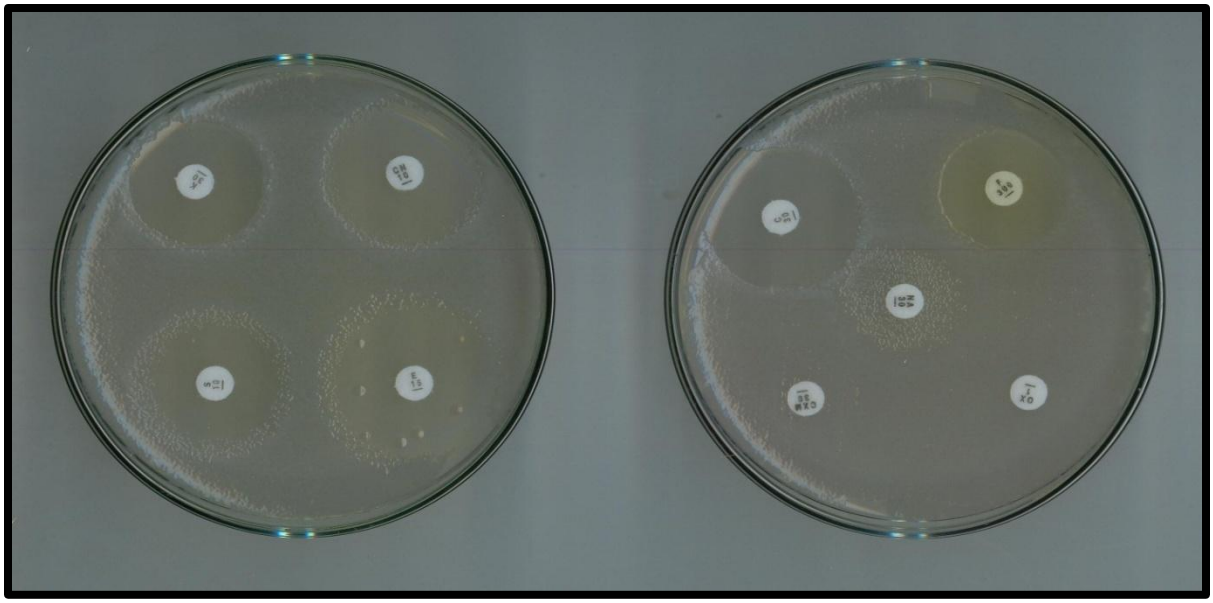
3.3 Selective antimicrobial activity test by means of antibiogram method

The standard disc diffusion test was done with all the provided antibiotics (Table 3) against five clinical and isolates of five selected environmental strains to identify their resistance pattern. The interpretive categories were defined according to the zone diameter of inhibition.

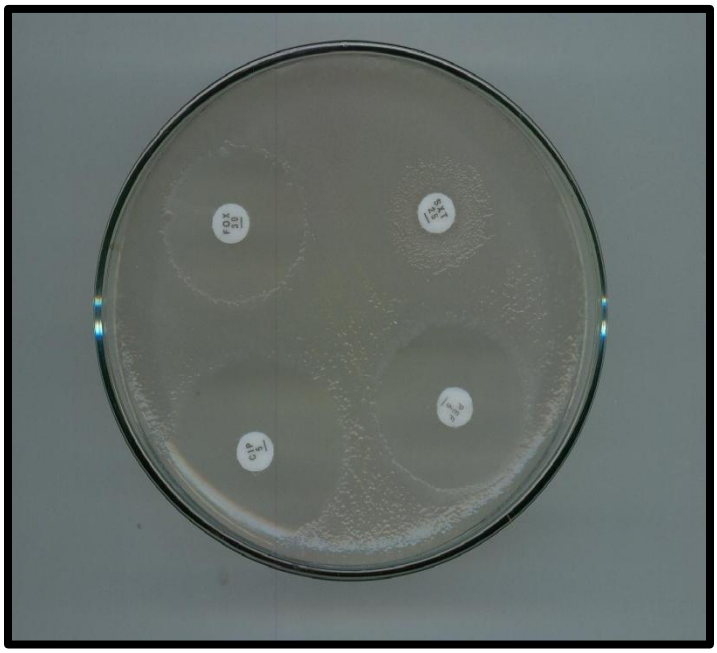
All the clinical strains showed significant susceptibility to all the antibiotics except for OX1 (Table 9). *Salmonella typhi* showed the maximum level of susceptibility to C30, which was around 34mm in diameter, whereas, both *E. coli* K12 and *Bacillus cereus* showed highest levels of susceptibility to CIP5 which was measured to be 40mm and 28mm in diameter respectively. *Bacillus cereus* also showed the similar level of susceptibility (28mm in diameter) to E15 as well. In case of *Shigella flexneri*, the utmost level of susceptibility was observed when CXM30 and FOX30 were applied in the disc diffusion test (26mm in diameter). In case of *Staphylococcus aureus*, the maximal level of vulnerability was to PEF5, which was around 32mm in diameter. Antibiotic susceptibility test results for clinical strains are represented in Table 9 and Figures 6, 7, 8, 9, and 10 below.

Table 9: Antibiotic susceptibility test results for clinical strains

Antibiotics	Clinical Strains				
	S. typhi	***E. coli* K12**	***B. cereus***	***S. flexneri***	***S. aureus***
SXT 25	33 mm	30 mm	9 mm	0 mm	28 mm
FOX 30	32 mm	28 mm	24 mm	26 mm	30 mm
PEF 5	22 mm	32 mm	25 mm	0 mm	32 mm
CIP 5	27 mm	40 mm	28 mm	16 mm	28 mm
E 15	8 mm	9 mm	28 mm	20 mm	9 mm
CN 10	25 mm	20 mm	25 mm	22 mm	20 mm
K 30	25 mm	19 mm	24 mm	24 mm	20 mm
S 10	17 mm	14 mm	20 mm	0 mm	14 mm
CXM 30	26 mm	25 mm	0 mm	26 mm	26 mm
NA 30	0 mm	24 mm	0 mm	0 mm	25 mm
OX 1	0 mm	0 mm	0 mm	0 mm	0 mm
C 30	34 mm	24 mm	26 mm	16 mm	26 mm
F 300	23 mm	26 mm	22 mm	24 mm	24 mm

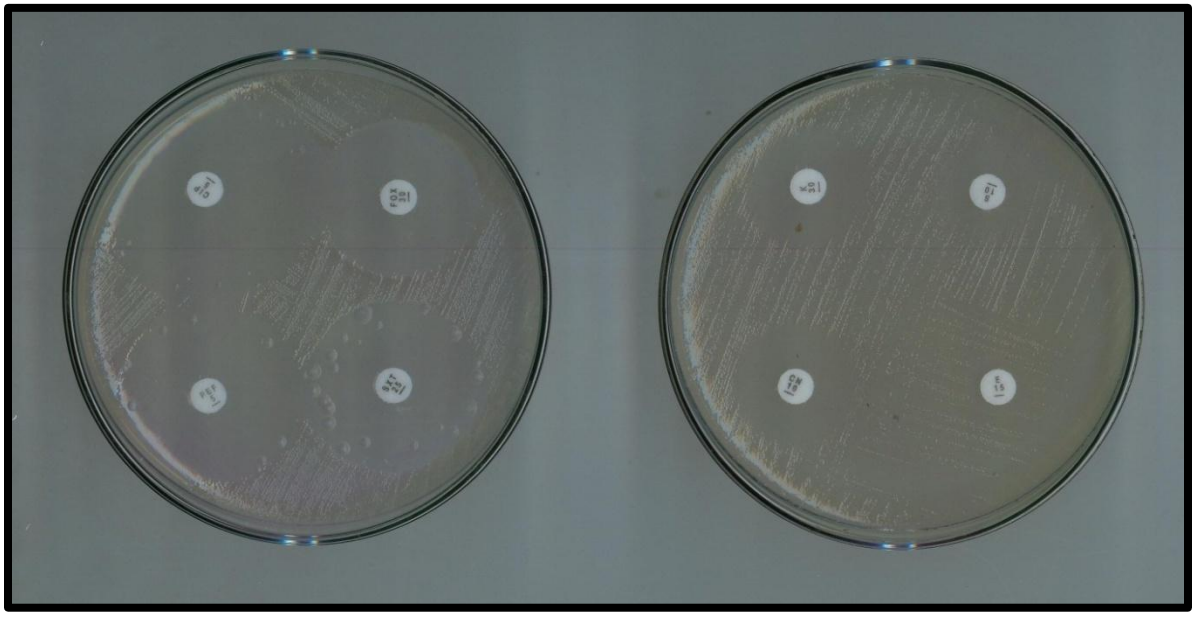


(a)

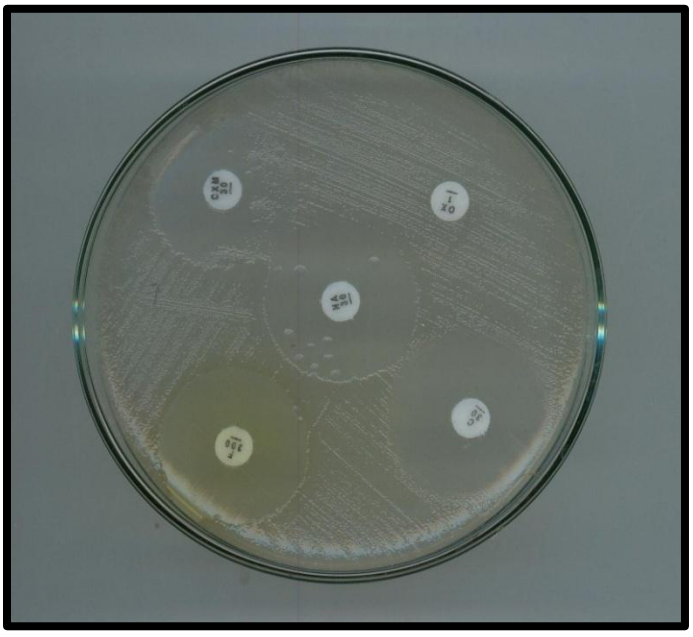


(b)

Figure 6: (a) and **(b)** Effect of provided antibiotics on clinical strain of *B. cereus*

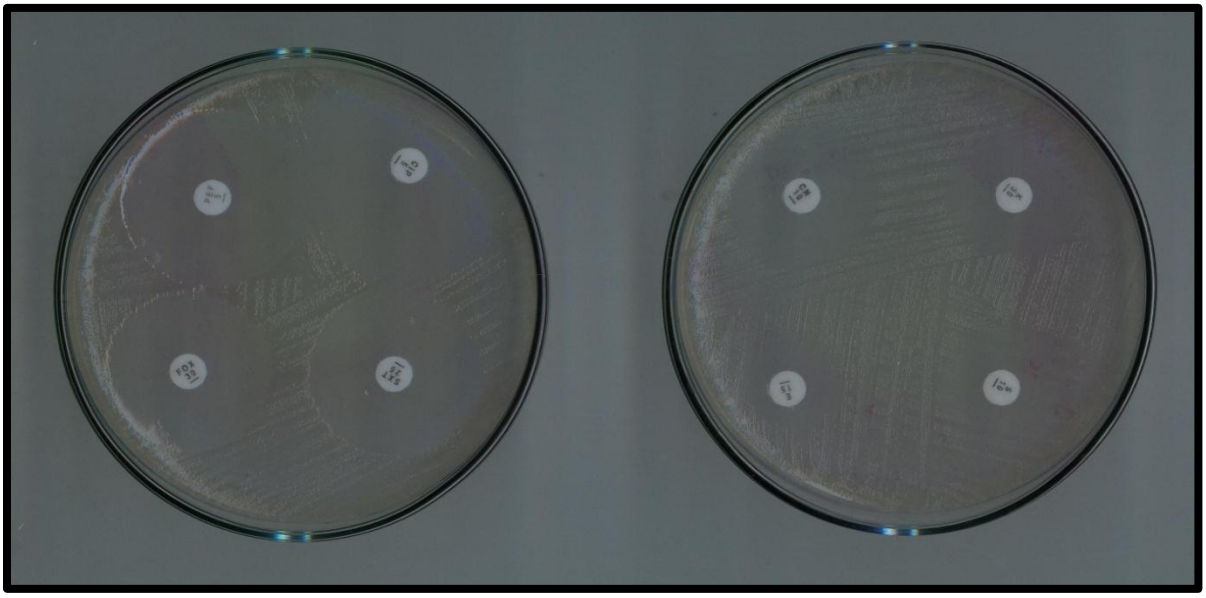


(a)

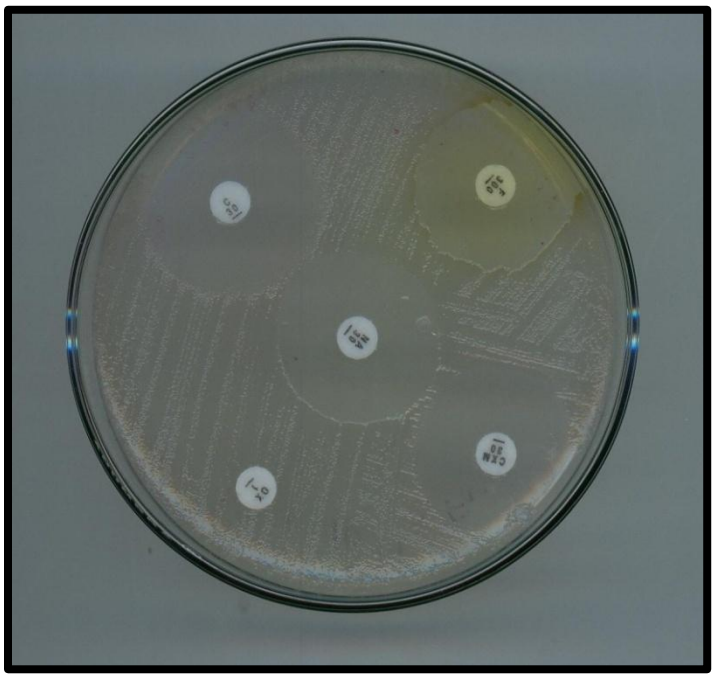


(b)

Figure 7: (a) and **(b)** Outcome of given antibiotics on clinical strain of *E. coli* K12

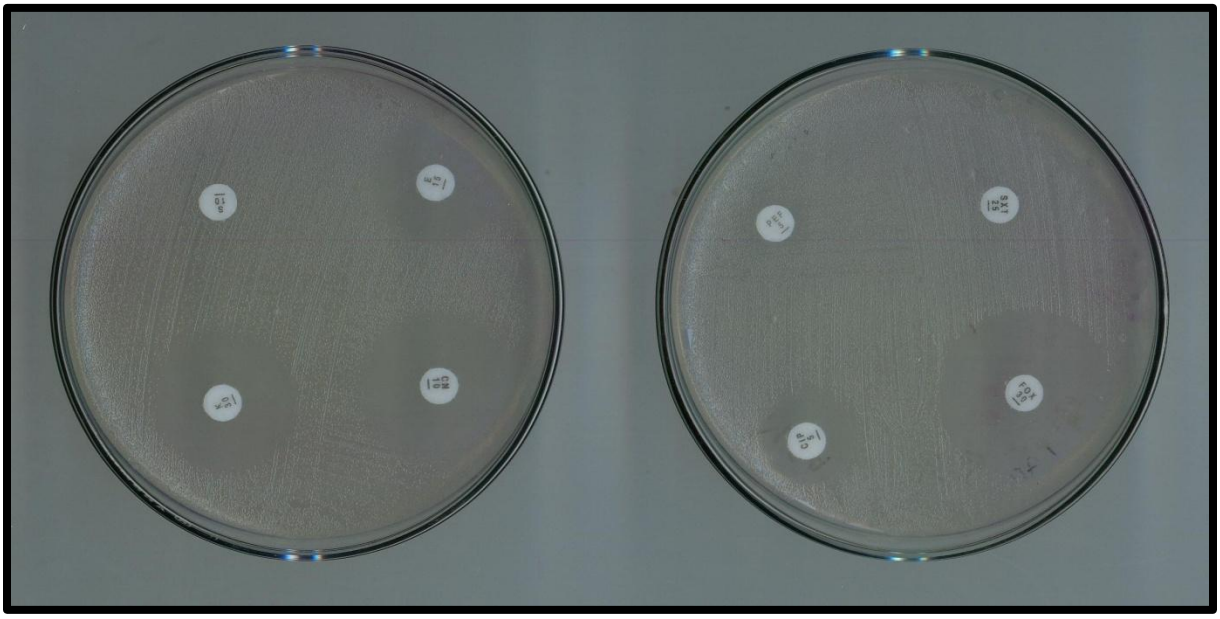


(a)

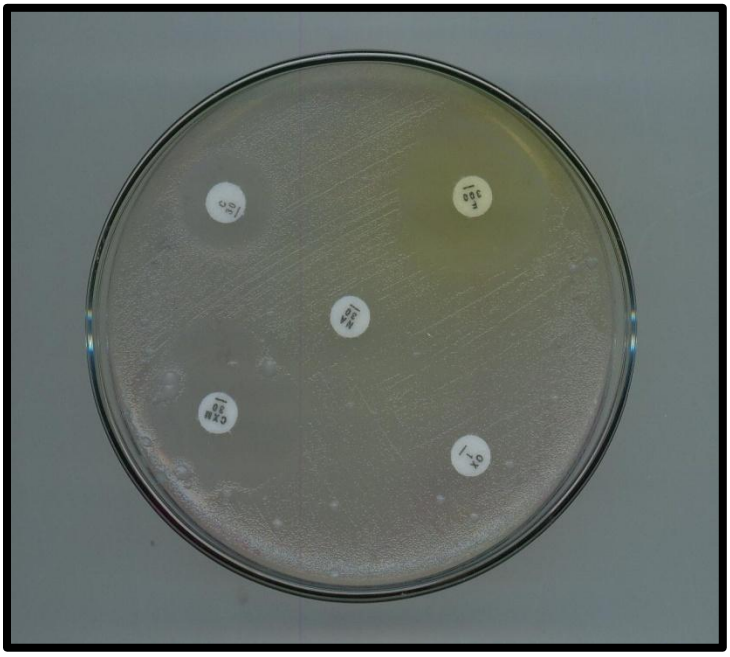


(b)

Figure 8: (a) and **(b)** Susceptibility pattern of supplied antibiotics on clinical strain of *S. aureus*

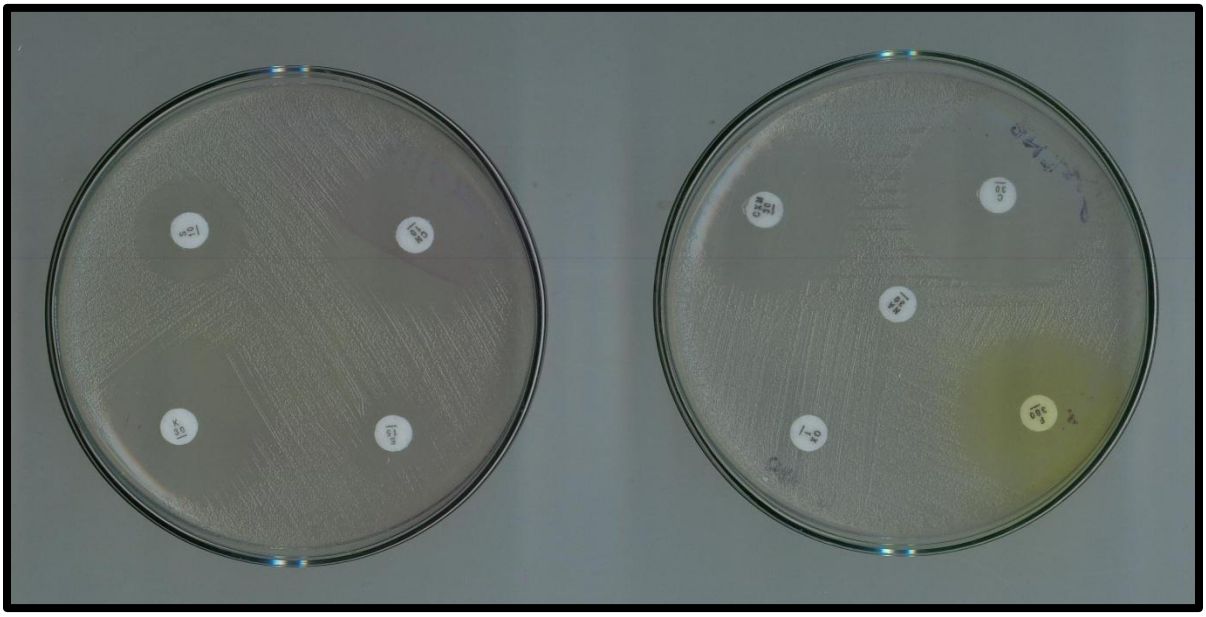


(a)

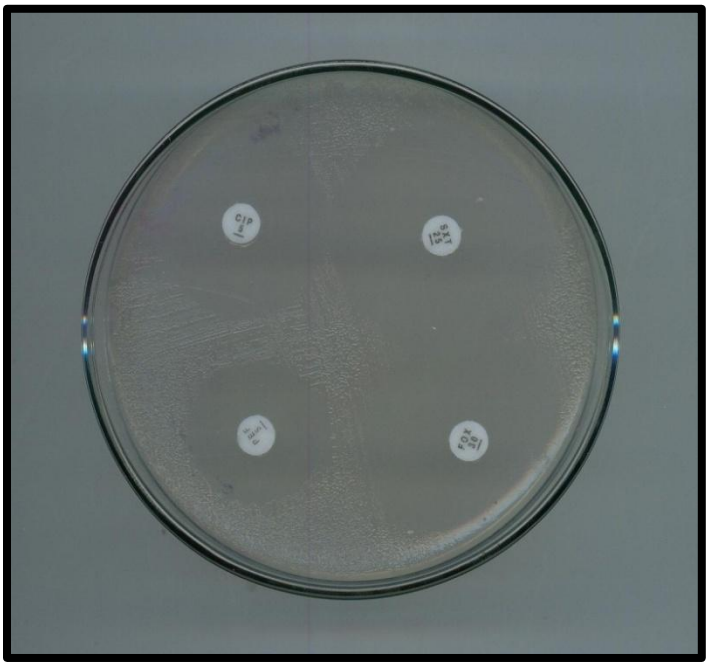


(b)

Figure 9: (a) and **(b)** Effect of provided antibiotics on clinical strain of *S. flexneri*



(a)



(b)

Figure 10: (a) and **(b)** Effectiveness of the applied antibiotics on clinical strain of *S. typhi*

A significant level of resistance was observed when all the standard antibiotics were applied against several isolates of selected environmental strains that were *Bacillus cereus* collected from salad sample and human sewage sample, *Shigella flexneri* collected from chicken feces, and *Salmonella typhi* collected from human sewage sample. *E. coli* was collected from salad sample and bovine sample (cow dung), and *Staphylococcus aureus* was collected from human sewage sample and chicken feces. They all showed resistance to at least one or more than one antibiotics.

In case of *B. cereus* collected from salad sample, the isolate S3 showed resistance to the antibiotic FOX30, and the isolate S6 was resistant to PEF5 and F300, whereas, the clinical strain of *B. cereus* showed significant zone of inhibition to these same antibiotics, which were measured to be 24mm, 25mm and 22mm in diameter respectively (Table 10). The isolate H1 of *B. cereus* collected from human sewage sample showed resistance to the antibiotic PEF5 and H2 showed resistance to CIP5 and S10. On the other hand their clinical strain showed zone of inhibition to these same antibiotics which were 25mm, 28mm, and 20mm in diameter respectively (Table 11).

The isolate C3 of *S. flexneri* collected from chicken feces showed resistance against two different antibiotics, FOX30 and E15, whereas the susceptibility of their clinical strain to these antibiotics was 26mm and 20mm in diameter respectively (Table 12). In case of *S. typhi*, which was collected from human sewage sample, the isolate H1 showed resistance to the antibiotics FOX30, E15, and S10, and the isolate H3 showed resistance to the antibiotics SXT25, PEF5, E15, and C30. The clinical strain of *S. typhi* was vulnerable to all these antibiotics (Table 13).

The clinical strain of *E. coli* was significantly vulnerable to all the antibiotics, yet three different isolates of *E. coli* collected from salad sample showed resistance to three different antibiotics each (Table 14) and four isolates collected from the bovine sample (cow dung) showed resistance to three and four different antibiotics each (Table 15).

In case of environmental strain of *Staphylococcus aureus*, the result was astounding. Its clinical strain was susceptible to all the test antibiotics but one, having large zones of inhibition measured to be as high as 28-32mm in diameter and yet different isolates of *S. aureus* collected from human sewage sample (Table 16) and chicken feces (Table 17) showed resistance to as many as four and eight different antibiotics as shown below.

Table 10: Antibiotic susceptibility test results for *B. cereus* collected from salad sample

Antibiotics	***B. cereus* From Salad Sample**					
	***B. cereus* S1**	***B. cereus* S2**	***B. cereus* S3**	***B. cereus* S4**	***B. cereus* S5**	***B. cereus* S6**
SXT 25	26 mm	8 mm	10 mm	14 mm	11 mm	17 mm
FOX 30	23 mm	18 mm	Resistant	9 mm	16 mm	26 mm
PEF 5	14 mm	20 mm	15 mm	18 mm	21 mm	Resistant
CIP 5	17 mm	12 mm	13 mm	17 mm	20 mm	18 mm
E15	15 mm	20 mm	11 mm	24 mm	7 mm	22 mm
CN 10	24 mm	23 mm	17 mm	11 mm	14 mm	20 mm
K 30	20 mm	28 mm	18 mm	24 mm	24 mm	19 mm
S 10	20 mm	18 mm	18 mm	21 mm	10 mm	16 mm
CXM 30	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
NA 30	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
OX 1	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
C 30	23 mm	19 mm	15 mm	28 mm	31 mm	24 mm
F 300	18 mm	9 mm	16 mm	13 mm	7 mm	Resistant

Table 11: Antibiotic susceptibility test results for *B. cereus* collected from human sewage sample

Antibiotics	***B. cereus* From Human Sewage Sample**					
	***B. cereus* H1**	***B. cereus* H2**	***B. cereus* H3**	***B. cereus* H4**	***B. cereus* H5**	***B. cereus* H6**
SXT 25	9 mm	11 mm	7 mm	18 mm	21 mm	13 mm
FOX 30	11 mm	9 mm	5 mm	7 mm	10 mm	16 mm
PEF 5	Resistant	13 mm	10 mm	14 mm	17 mm	22 mm
CIP 5	19 mm	Resistant	16 mm	24 mm	21 mm	20 mm
E15	14 mm	19 mm	15 mm	21 mm	11 mm	20 mm
CN 10	9 mm	22 mm	12 mm	18 mm	26 mm	14 mm
K 30	18 mm	17 mm	20 mm	18 mm	18 mm	18 mm
S 10	21 mm	Resistant	23 mm	26 mm	20 mm	24 mm
CXM 30	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
NA 30	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
OX 1	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
C 30	16 mm	14 mm	13 mm	7 mm	10 mm	8 mm
F 300	12 mm	11 mm	14 mm	20 mm	13 mm	17 mm

Table 12: Antibiotic susceptibility test results for *S. flexneri* collected from chicken feces

Antibiotics	***S. flexneri* From Chicken Feces**						
	***S. flexneri* C1**	***S. flexneri* C2**	***S. flexneri* C3**	***S. flexneri* C4**	***S. flexneri* C5**	***S. flexneri* C6**	***S. flexneri* C7**
SXT 25	15 mm	12 mm	0 mm	21 mm	17 mm	19 mm	14 mm
FOX 30	24 mm	19 mm	Resistant	16 mm	20 mm	18 mm	12 mm
PEF 5	20 mm	16 mm	8 mm	22 mm	22 mm	14 mm	19 mm
CIP 5	17 mm	19 mm	12 mm	25 mm	21 mm	31 mm	24 mm
E15	22 mm	11 mm	Resistant	15 mm	19 mm	23 mm	15 mm
CN 10	22 mm	13 mm	9 mm	18 mm	21 mm	19 mm	13 mm
K 30	23 mm	16 mm	21 mm	32 mm	24 mm	21 mm	17 mm
S 10	26 mm	11 mm	8 mm	13 mm	17 mm	17 mm	19 mm
CXM 30	20 mm	20 mm	23 mm	20 mm	27 mm	24 mm	22 mm
NA 30	19 mm	17 mm	0 mm	13 mm	30 mm	11 mm	16 mm
OX 1	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
C 30	17 mm	19 mm	9 mm	14 mm	16 mm	11 mm	7 mm
F 300	25 mm	11 mm	10 mm	6 mm	11 mm	28 mm	22 mm

Table 13: Antibiotic susceptibility test results for *S. typhi* collected from human sewage sample

Antibiotics	***S. typhi* From Human sewage sample**					
	***S. typhi* H1**	***S. typhi* H2**	***S. typhi* H3**	***S. typhi* H4**	***S. typhi* H5**	***S. typhi* H6**
SXT 25	18 mm	16 mm	Resistant	19 mm	15 mm	13 mm
FOX 30	Resistant	21 mm	12 mm	17 mm	23 mm	21 mm
PEF 5	26 mm	18 mm	Resistant	22 mm	20 mm	21 mm
CIP 5	23 mm	22 mm	13 mm	20 mm	19 mm	17 mm
E15	Resistant	11 mm	Resistant	9 mm	13 mm	13 mm
CN 10	13 mm	17 mm	15 mm	19 mm	17 mm	15 mm
K 30	17 mm	9 mm	10 mm	15 mm	13 mm	7 mm
S 10	Resistant	11 mm	16 mm	12 mm	11 mm	14 mm
CXM 30	22 mm	23 mm	16 mm	11 mm	28 mm	21 mm
NA 30	29 mm	21 mm	0 mm	19 mm	22 mm	17 mm
OX 1	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
C 30	14 mm	11 mm	Resistant	11 mm	14 mm	12 mm
F 300	11 mm	11 mm	9 mm	7 mm	12 mm	8 mm

Table 14: Antibiotic susceptibility test results for *E. coli* collected from salad sample

Antibiotics	*E. coli* From Salad Sample								
	***E. coli* S1**	***E. coli* S2**	***E. coli* S3**	***E. coli* S4**	***E. coli* S5**	***E. coli* S6**	***E. coli* S7**	***E. coli* S8**	***E. coli* S9**
SXT 25	8 mm	12 mm	Resistant	14 mm	14 mm	10 mm	12 mm	19 mm	12 mm
FOX 30	6 mm	19 mm	8 mm	16 mm	19 mm	12 mm	17 mm	21 mm	13 mm
PEF 5	Resistant	13 mm	12 mm	15 mm	22 mm	12 mm	14 mm	12 mm	28 mm
CIP 5	20 mm	24 mm	28 mm	24 mm	18 mm	21 mm	19 mm	20 mm	22 mm
E15	18 mm	14 mm	9 mm	14 mm	21 mm	10 mm	11 mm	13 mm	Resistant
CN 10	Resistant	10 mm	8 mm	14 mm	19 mm	17 mm	13 mm	18 mm	17 mm
K 30	14 mm	18 mm	Resistant	22 mm	22 mm	27 mm	24 mm	22 mm	Resistant
S 10	22 mm	24 mm	13 mm	19 mm	15 mm	28 mm	24 mm	20 mm	21 mm
CXM 30	Resistant	18 mm	Resistant	17 mm	11 mm	14 mm	28 mm	19 mm	15 mm
NA 30	10 mm	13 mm	6 mm	11 mm	9 mm	8 mm	18 mm	15 mm	9 mm
OX 1	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
C 30	13 mm	9 mm	10 mm	13 mm	15 mm	18 mm	19 mm	8 mm	14 mm
F 300	16 mm	14 mm	13 mm	19 mm	11 mm	15 mm	13 mm	11 mm	12 mm

Table 15: Antibiotic susceptibility test results for *E. coli* collected from bovine sample

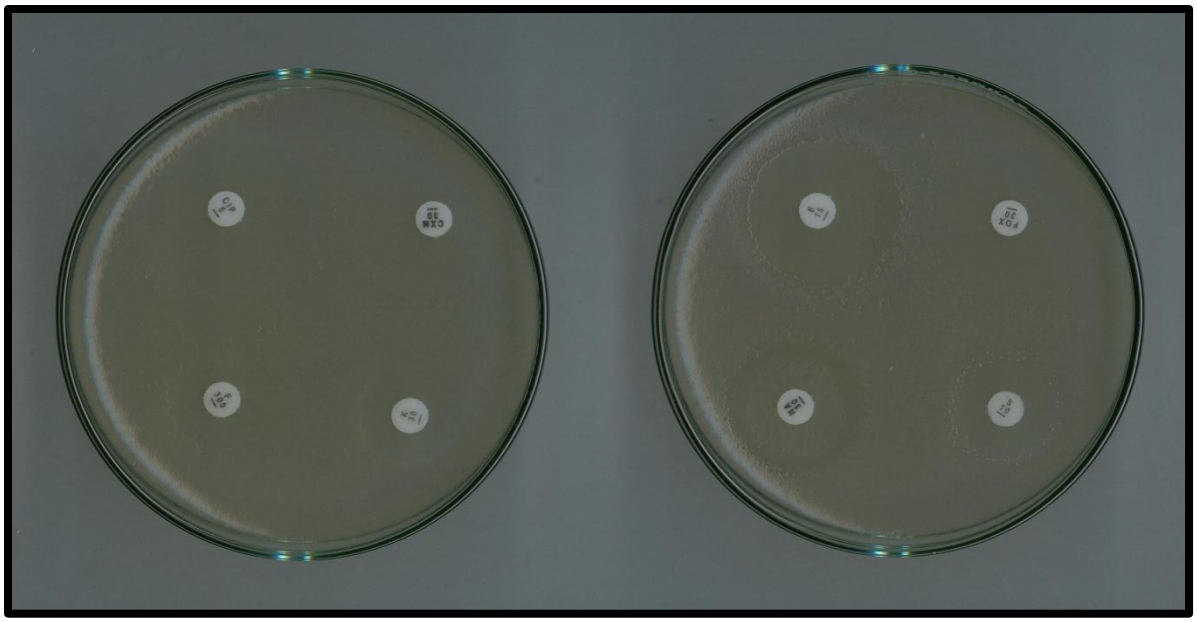
Antibiotics	*E. coli* From Bovine Sample								
	***E. coli* B1**	***E. coli* B2**	***E. coli* B3**	***E. coli* B4**	***E. coli* B5**	***E. coli* B6**	***E. coli* B7**	***E. coli* B8**	***E. coli* B9**
SXT 25	7 mm	12 mm	Resistant	16 mm	Resistant	18 mm	12 mm	10 mm	14 mm
FOX 30	5 mm	9 mm	12 mm	16 mm	8 mm	11 mm	16 mm	14 mm	17 mm
PEF 5	Resistant	15 mm	Resistant	17 mm	12 mm	10mm	19 mm	14 mm	12 mm
CIP 5	16 mm	13 mm	8 mm	10 mm	28 mm	23 mm	10 mm	8 mm	14 mm
E15	Resistant	19 mm	9 mm	11 mm	9 mm	10 mm	17 mm	Resistant	12 mm
CN 10	12 mm	12 mm	15 mm	11 mm	8 mm	15 mm	18 mm	Resistant	14 mm
K 30	20 mm	18 mm	18 mm	10 mm	Resistant	22 mm	11 mm	16 mm	19 mm
S 10	Resistant	18 mm	13 mm	17 mm	13 mm	24 mm	11 mm	20 mm	16 mm
CXM 30	0 mm	13 mm	16 mm	16 mm	20 mm	22 mm	19 mm	Resistant	14 mm
NA 30	0 mm	0 mm	Resistant	0 mm	6 mm	9 mm	8 mm	10 mm	17 mm
OX 1	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
C 30	13 mm	15 mm	16 mm	9 mm	10 mm	22 mm	24 mm	Resistant	20 mm
F 300	20 mm	16 mm	14 mm	19 mm	13 mm	17 mm	20 mm	21 mm	18 mm

Table 16: Antibiotic susceptibility test results for *S. aureus* collected from human sewage sample

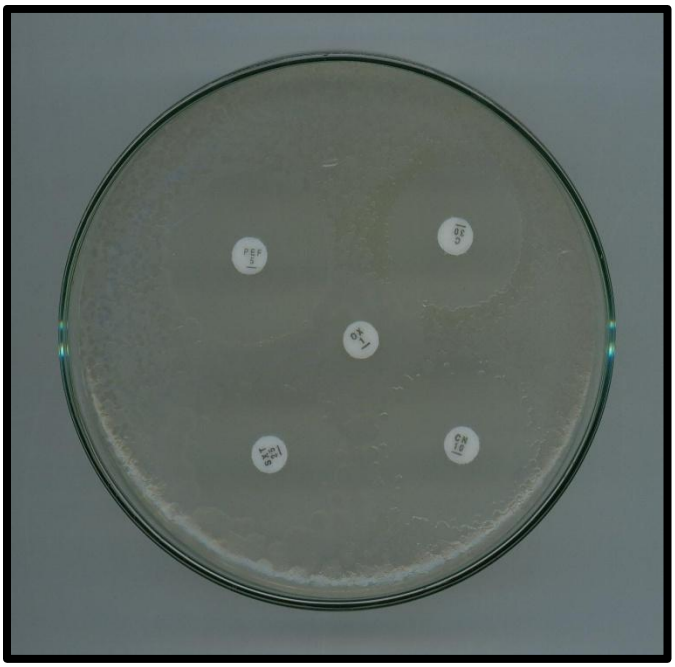
Antibiotics	***S. aureus* From Human sewage sample**							
	***S. aureus* H1**	***S. aureus* H2**	***S. aureus* H3**	***S. aureus* H4**	***S. aureus* H5**	***S. aureus* H6**	***S. aureus* H7**	***S. aureus* H8**
SXT 25	18 mm	22 mm	12 mm	18 mm	17 mm	14 mm	18 mm	21 mm
FOX 30	17 mm	19 mm	14 mm	26 mm	21 mm	18 mm	28 mm	33 mm
PEF 5	24 mm	26 mm	8 mm	13 mm	17 mm	11 mm	15 mm	29 mm
CIP 5	11 mm	16 mm	6 mm	18 mm	21 mm	13 mm	13 mm	25 mm
E15	16 mm	11 mm	7 mm	22 mm	Resistant	8 mm	11 mm	11 mm
CN 10	21 mm	13 mm	5 mm	17 mm	Resistant	6 mm	13 mm	Resistant
K 30	19 mm	11 mm	11 mm	13 mm	19 mm	22 mm	12 mm	20 mm
S 10	24 mm	23 mm	11 mm	16 mm	22 mm	15 mm	28 mm	31 mm
CXM 30	20 mm	21 mm	15 mm	19 mm	26 mm	11 mm	24 mm	Resistant
NA 30	26 mm	21 mm	Resistant	13 mm	Resistant	9 mm	19 mm	17 mm
OX 1	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
C 30	19 mm	13 mm	28 mm	25 mm	11 mm	7 mm	18 mm	18 mm
F 300	11 mm	9 mm	23 mm	13 mm	Resistant	5 mm	17 mm	22 mm

Table 17: Antibiotic susceptibility test results for *S. aureus* collected from chicken feces

Antibiotics	***S. aureus* From Chicken Feces**							
	***S. aureus* C1**	***S. aureus* C2**	***S. aureus* C3**	***S. aureus* C4**	***S. aureus* C5**	***S. aureus* C6**	***S. aureus* C7**	***S. aureus* C8**
SXT 25	9 mm	11 mm	Resistant	17 mm	21 mm	19 mm	15 mm	22 mm
FOX 30	13 mm	17 mm	12 mm	16 mm	11 mm	25 mm	19 mm	11 mm
PEF 5	11 mm	9 mm	Resistant	28 mm	Resistant	17 mm	15 mm	26 mm
CIP 5	14 mm	11 mm	Resistant	19 mm	Resistant	15 mm	13 mm	15 mm
E15	13 mm	15 mm	Resistant	22 mm	12 mm	18 mm	11 mm	17 mm
CN 10	12 mm	18 mm	Resistant	10 mm	8 mm	14 mm	17 mm	15 mm
K 30	28 mm	13 mm	Resistant	7 mm	19 mm	21 mm	15 mm	13 mm
S 10	24 mm	16 mm	Resistant	24 mm	22 mm	19 mm	21 mm	11 mm
CXM 30	21 mm	19 mm	21 mm	26 mm	23 mm	21 mm	23 mm	17 mm
NA 30	28 mm	26 mm	Resistant	13 mm	17 mm	15 mm	19 mm	24 mm
OX 1	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
C 30	19 mm	16 mm	28 mm	21 mm	31 mm	33 mm	22 mm	25 mm
F 300	14 mm	22 mm	21 mm	11 mm	16 mm	19 mm	17 mm	19 mm

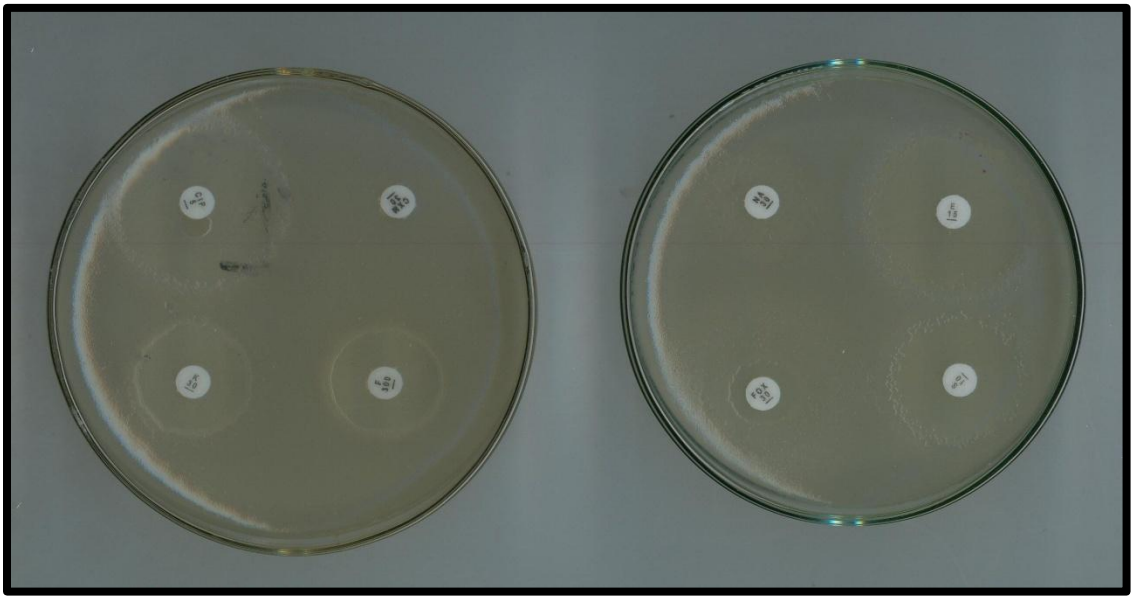


(a)

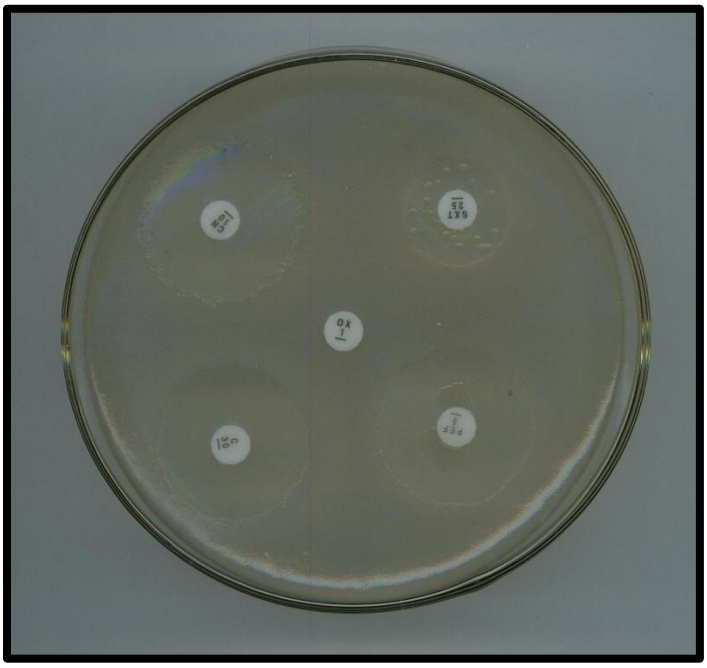


(b)

Figure 11: (a) and **(b)** Effect of provided antibiotics on *B. cereus* S3 isolate collected from salad sample

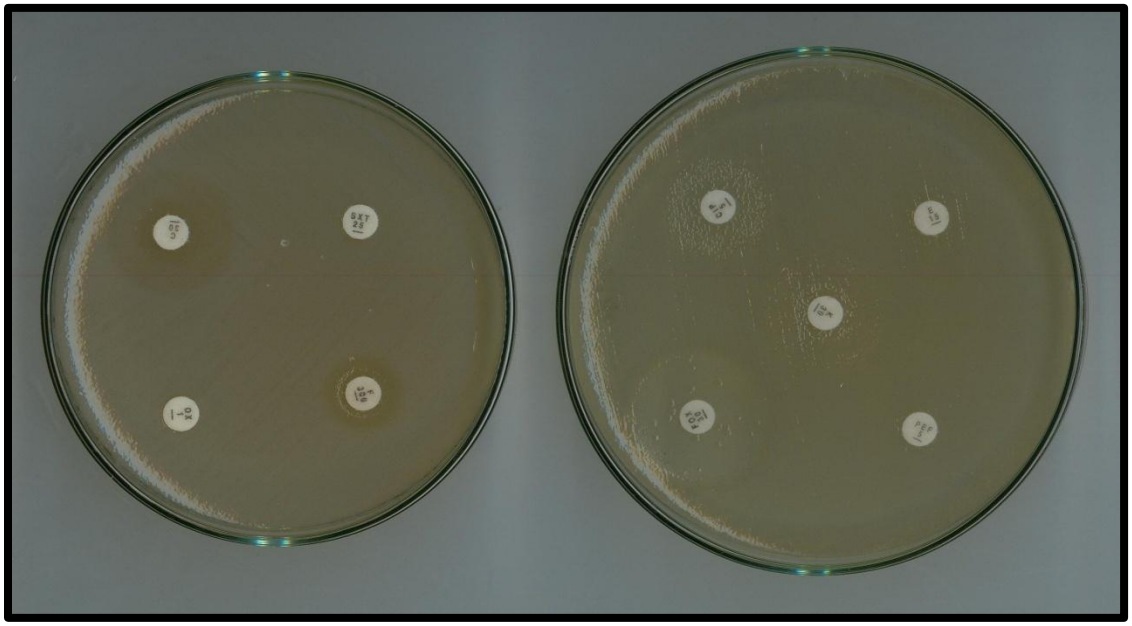


(a)

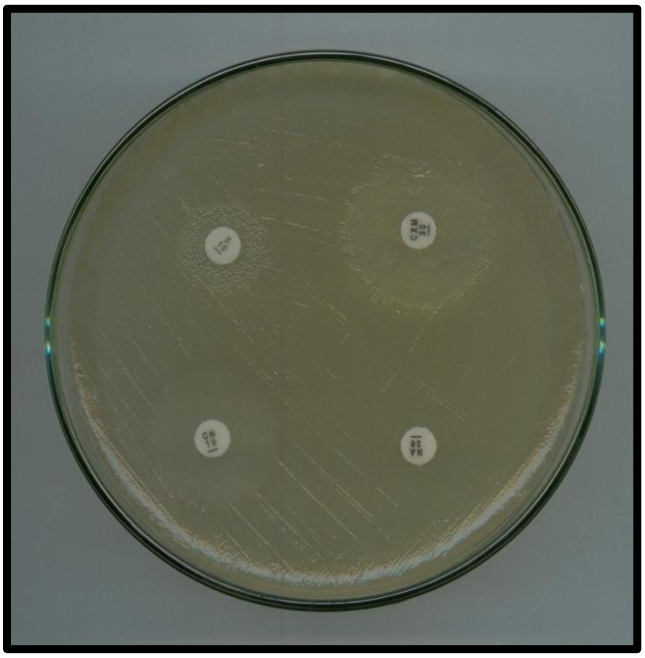


(b)

Figure 12: (a) and **(b)** Effect of provided antibiotics on *B. cereus* H2 isolate collected from human sewage sample

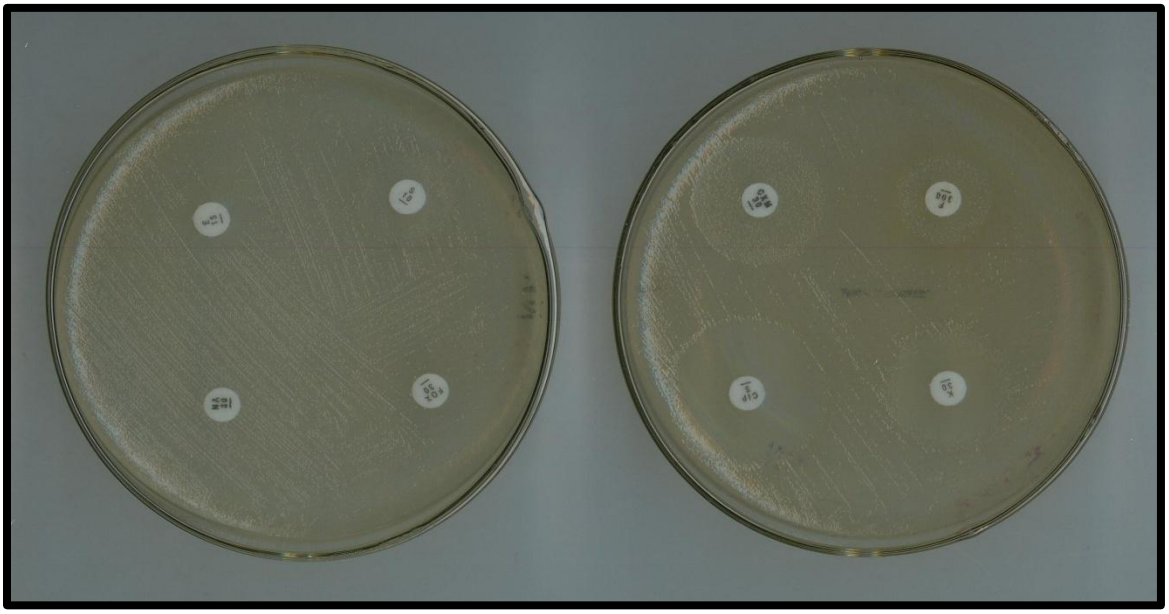


(a)

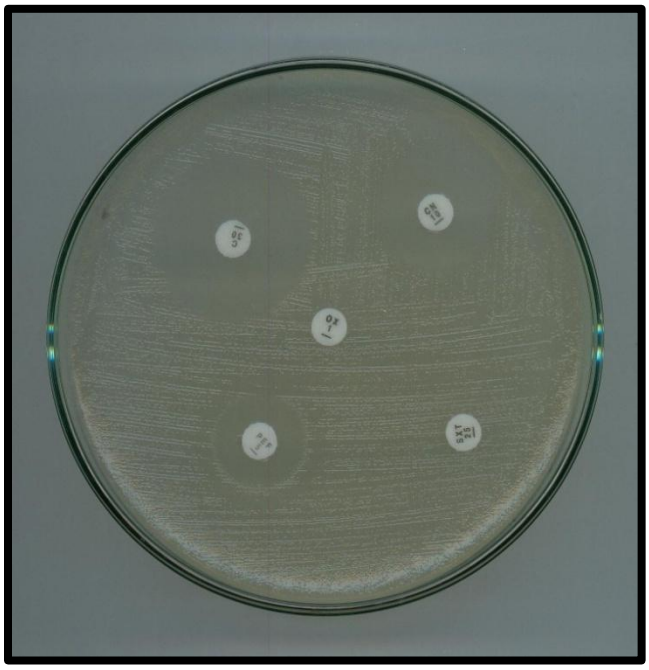


(b)

Figure 13: (a) and **(b)** Effectiveness of applied antibiotics on *S. typhi* H3 isolate collected from human sewage sample

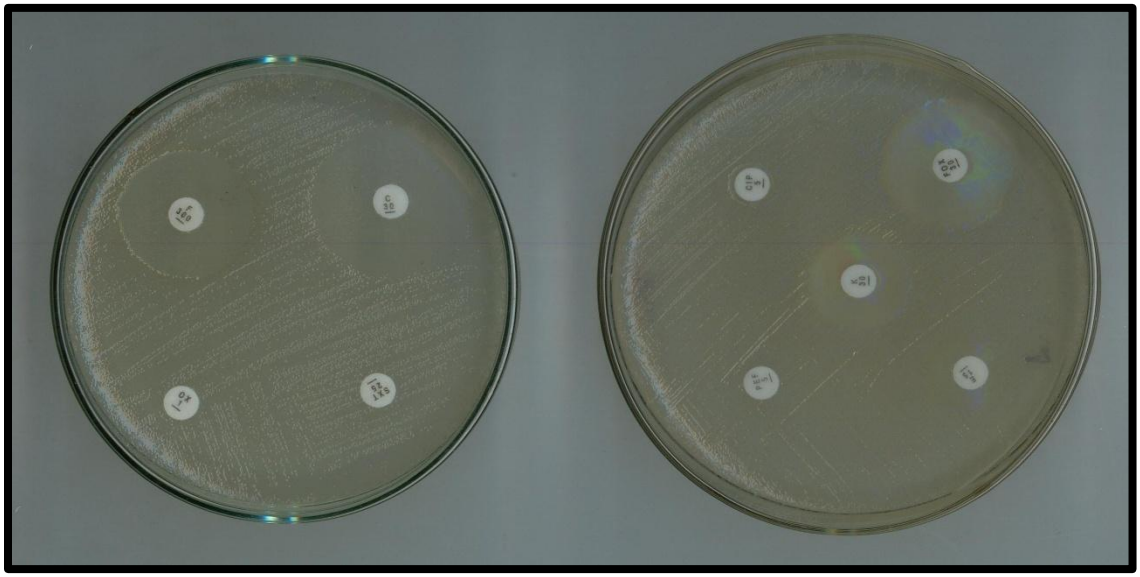


(a)



(b)

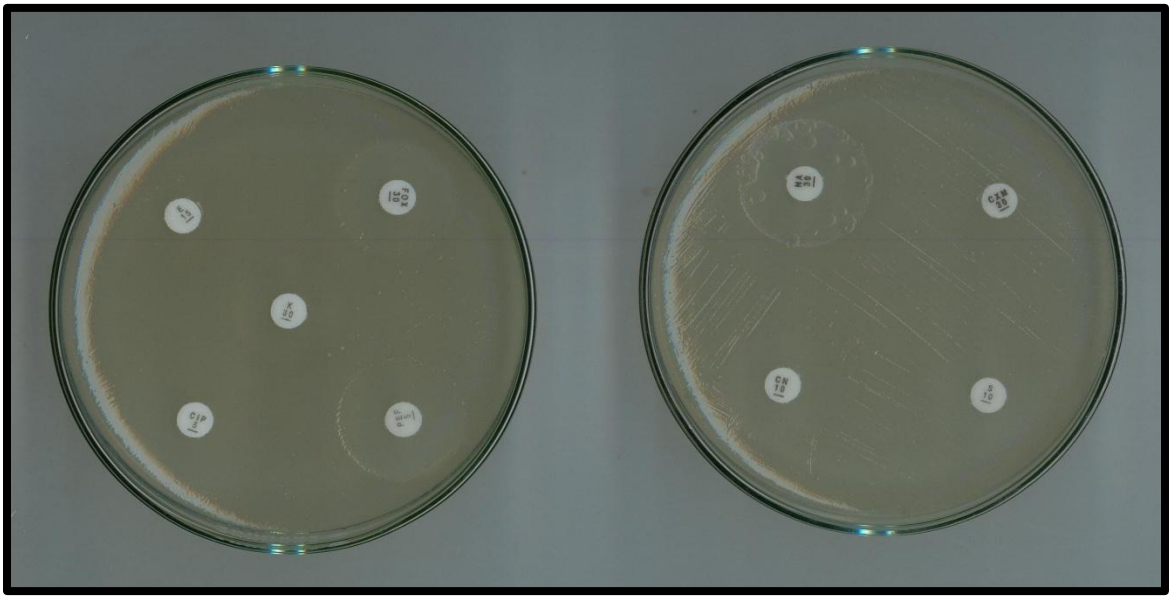
Figure 14: (a) and **(b)** Effect of provided antibiotics on *S. flexneri* C3 isolate collected from chicken feces



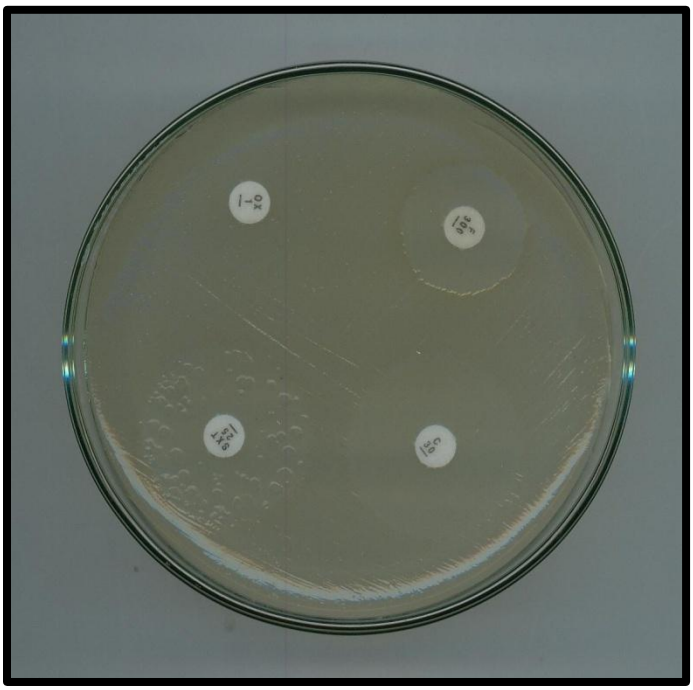
(a)

(b)

Figure 15: (a) and **(b)** Outcome of given antibiotics on *E. coli* B3 isolate collected from bovine sample

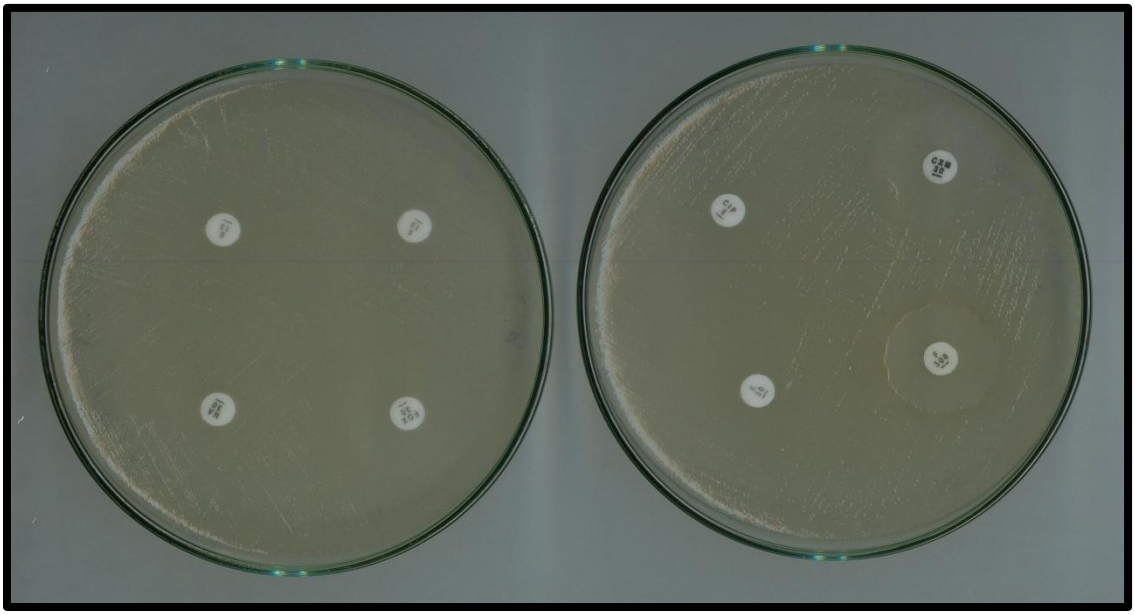


(a)

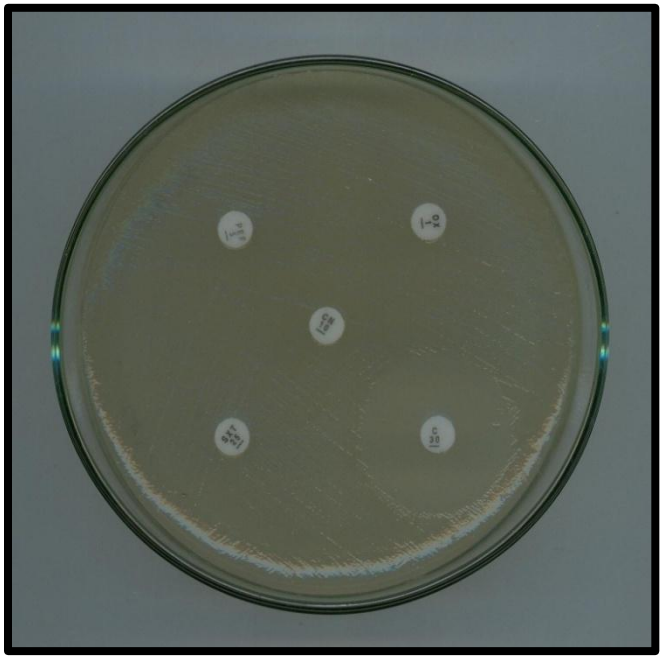


(b)

Figure 16: (a) and **(b)** Outcome of given antibiotics on *E. coli* S3 isolate collected from salad sample

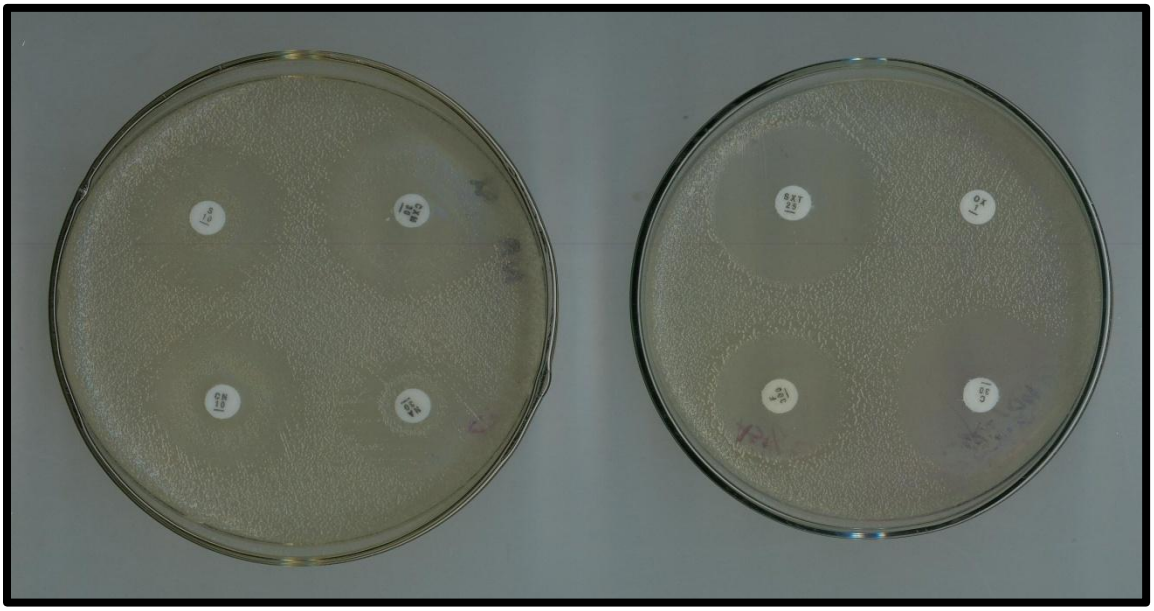


(a)



(b)

Figure 17: (a) and **(b)** Effectiveness of applied antibiotics on *S. aureus* C3 isolate collected from chicken feces



(a)

(b)

Figure 18: (a) and **(b)** Effectiveness of applied antibiotics on *S. aureus* H3 isolate collected from human sewage sample

3.4 Comparative analysis of antibiotic susceptibility profiles of clinical and environmental strains

A comparative study was carried out among clinical and environmental samples isolated from salad sample, human sewage sample, chicken feces, and bovine sample. Results indicate strong differences in the numbers of different environmental isolates and the clinical strains in terms of the development of resistance to standard antibiotic disc.

B. cereus isolates collected from salad sample showed resistance to the antibiotic FOX30, PEF5 and F300 whereas *B. cereus* collected from human sewage sample showed resistance to PEF5, CIP5, and S10 when compared to clinical isolates of *B. cereus* for the same antimicrobial tested. *S. flexneri* isolate collected from the feces of chicken showed resistance against two different antibiotics, FOX30 and E15, whereas for their clinical strains, its susceptibility to these antibiotics were moderately high. *E. coli* isolates collected from salad sample and bovine sample both showed resistance to two-three different antibiotics each. On the other hand, the clinical strain of *E. coli* was considerably vulnerable to those same antibiotics. A noticeable fact in the susceptibility test result of *S. typhi* was that its clinical strain showed maximum level of susceptibility to the antibiotic C30, according to section 3.3, and one of its environmental isolate namely H3 collected from human sewage sample showed resistance to this same antibiotic. In case of *Staphylococcus aureus*, its clinical strain was susceptible to all the antibiotics but one, and yet its environmental isolates collected both from human sewage sample and chicken feces showed resistance to as many as four and eight different antibiotics as presented in the figures below.

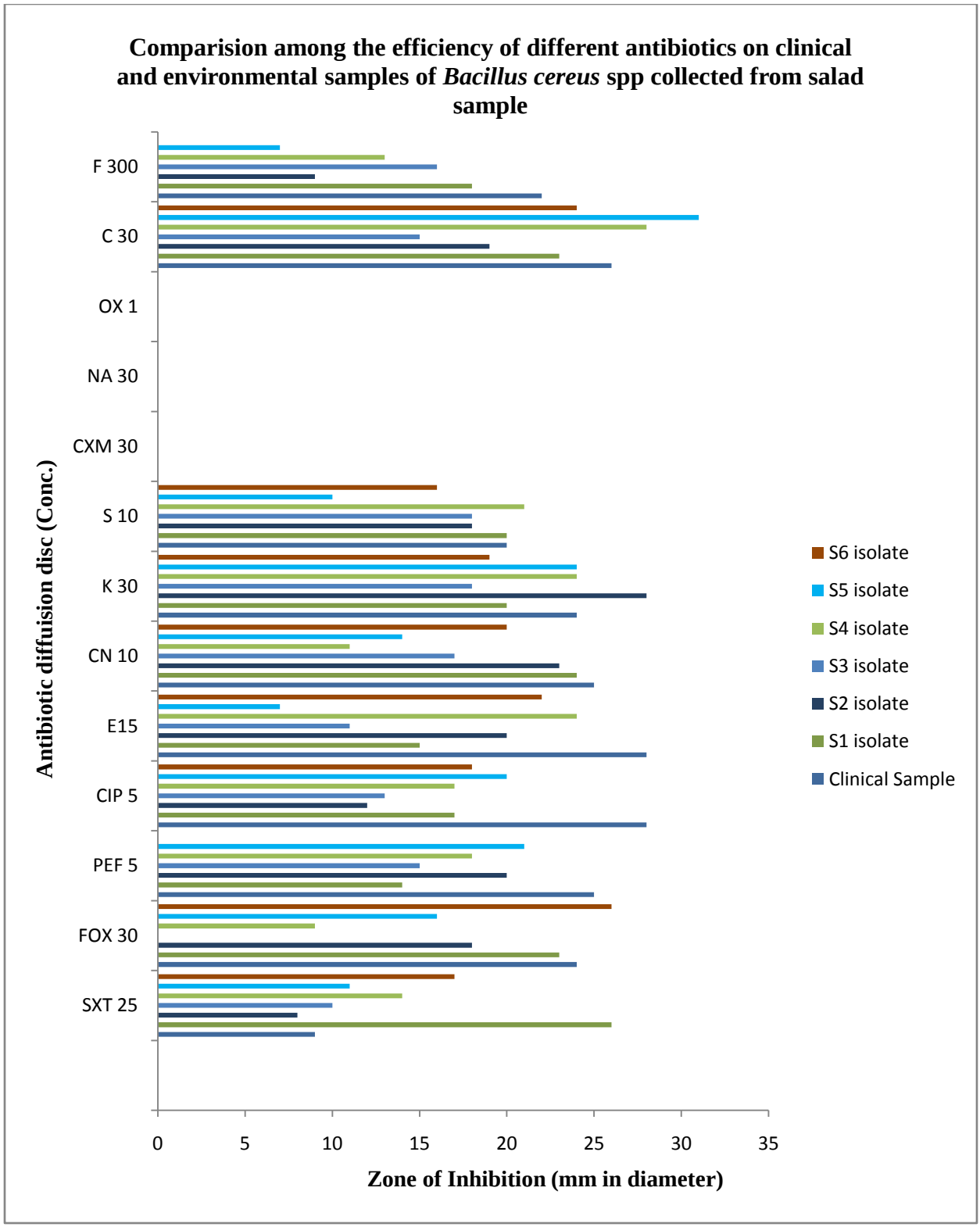


Figure 19: Comparison among the efficiency of different antibiotics on clinical and environmental samples of *B. cereus* spp collected from salad sample

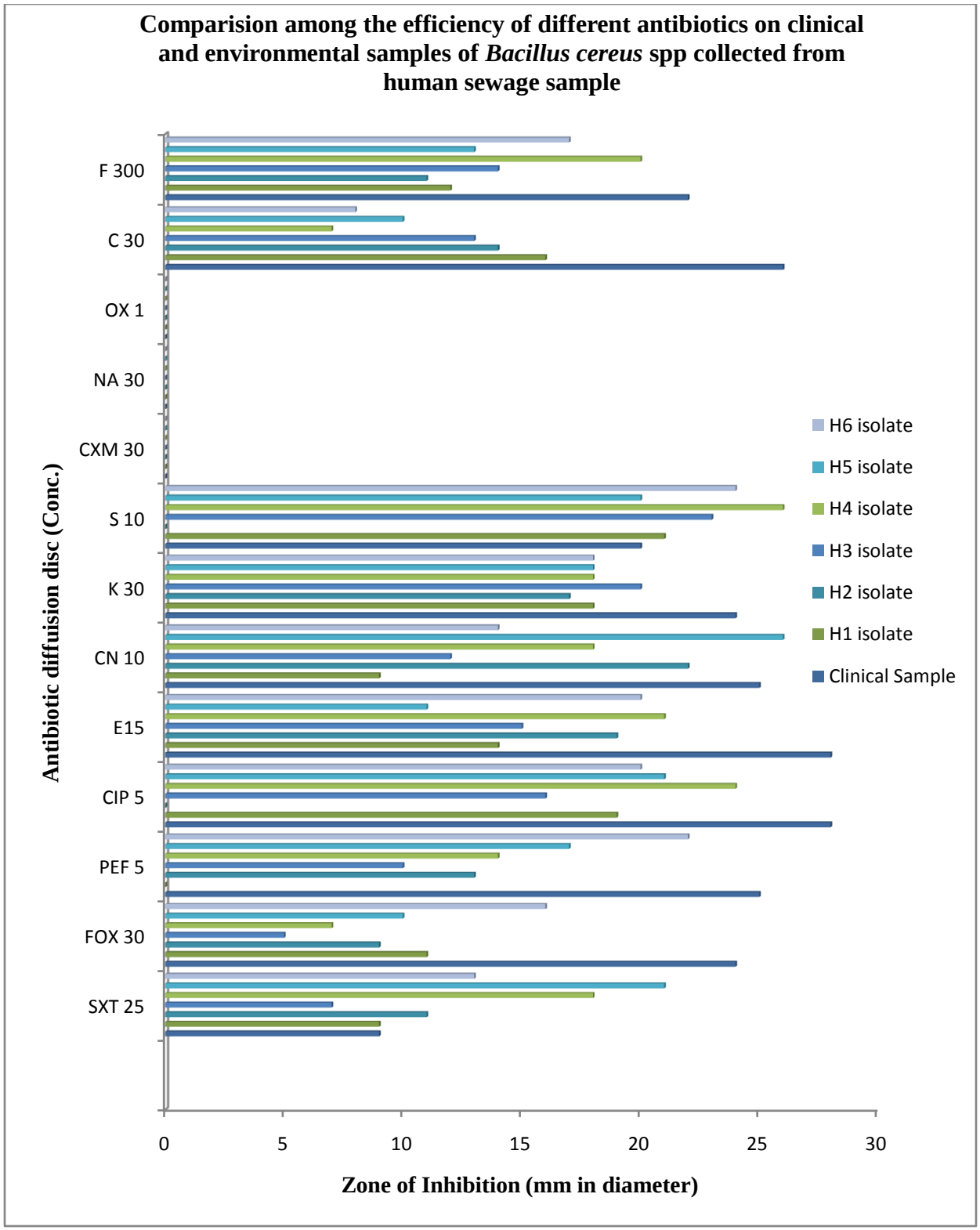


Figure 20: Comparison among the efficiency of different standard antibiotics on clinical and environmental samples of *B. cereus* spp collected from human sewage sample

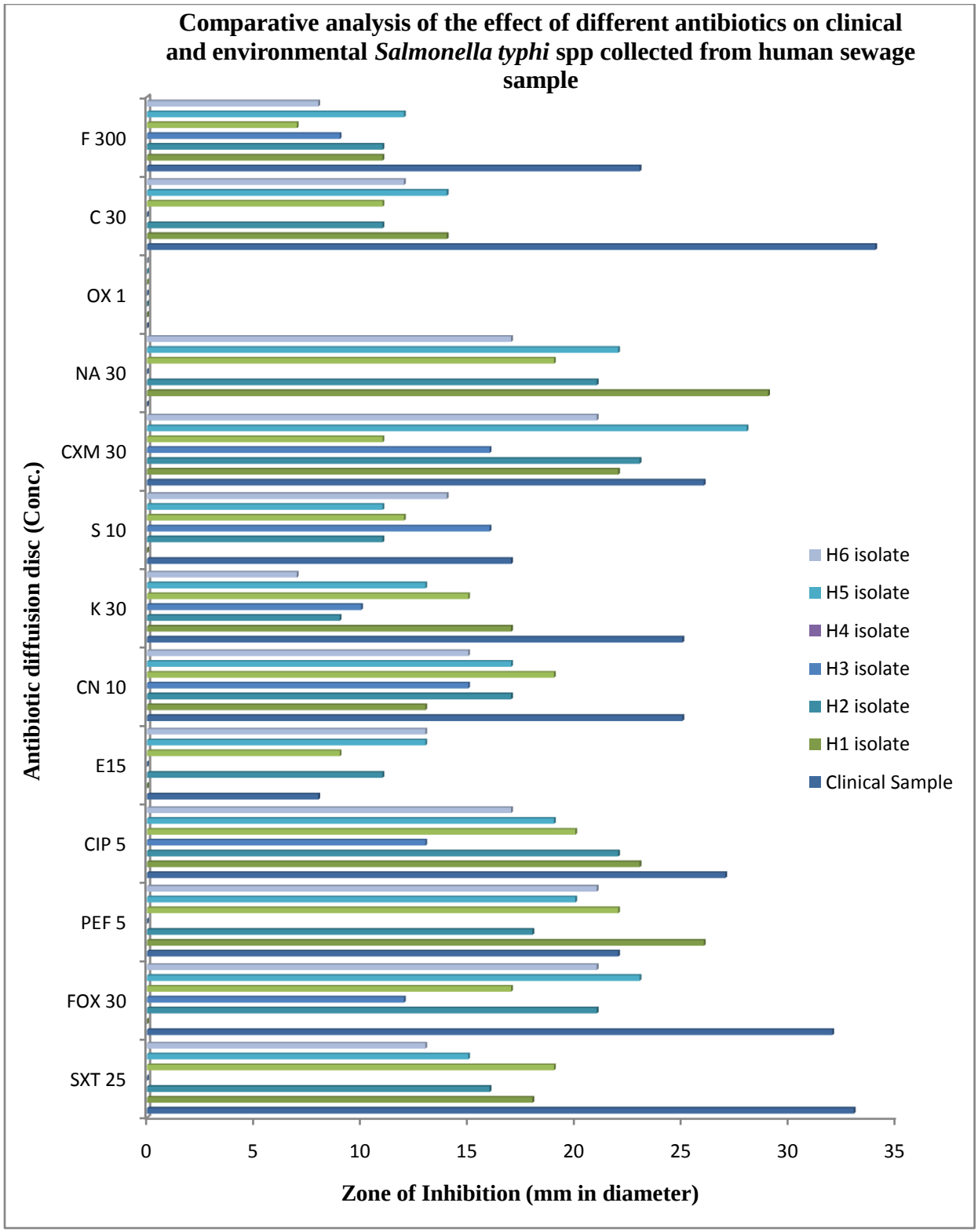


Figure 21: Comparative analysis of the effect of different antibiotics on clinical and environmental *Salmonella typhi* spp collected from human sewage sample

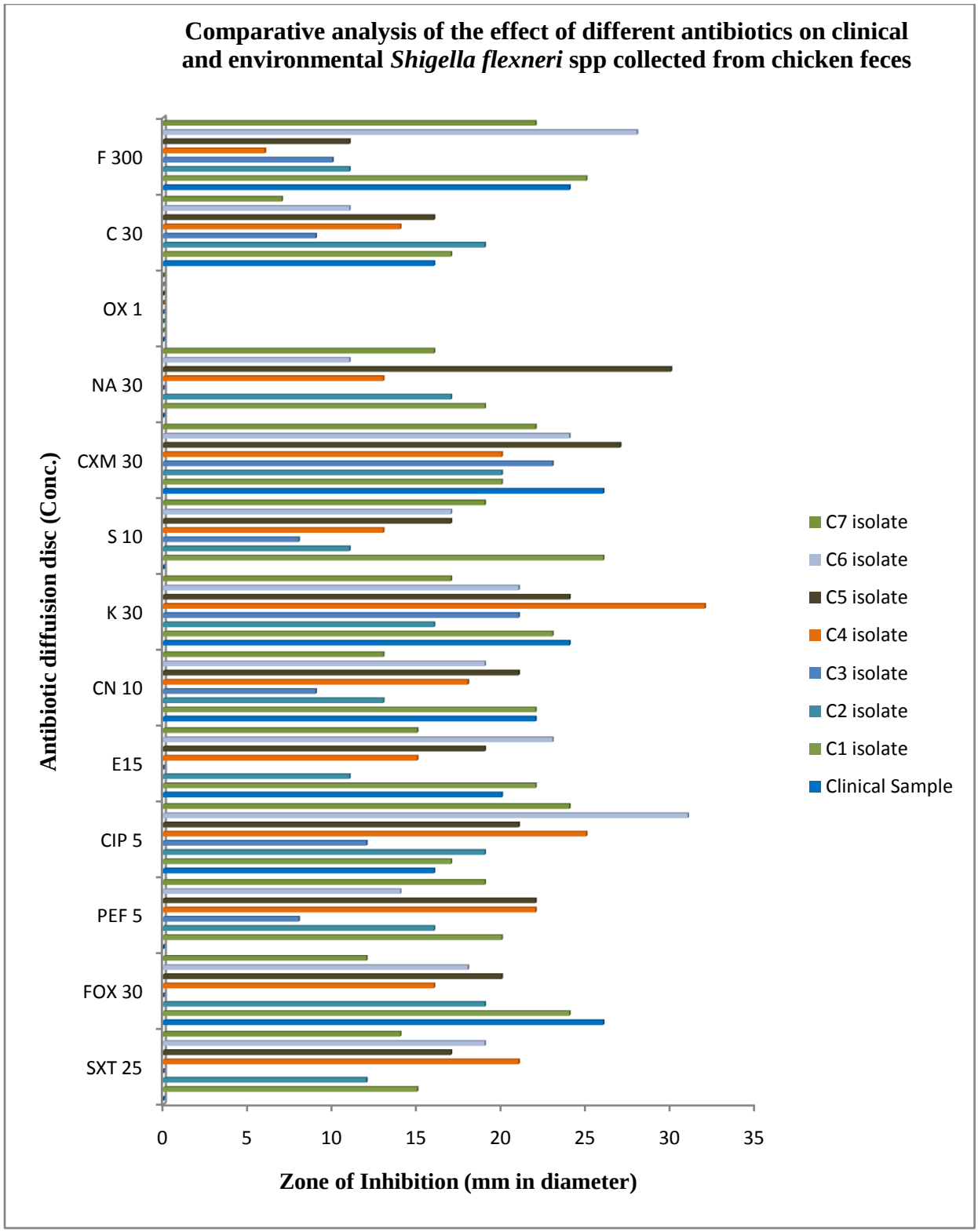


Figure 22: Comparative analysis of the effect of different antibiotics on clinical and environmental *Shigella flexneri* spp collected from chicken feces

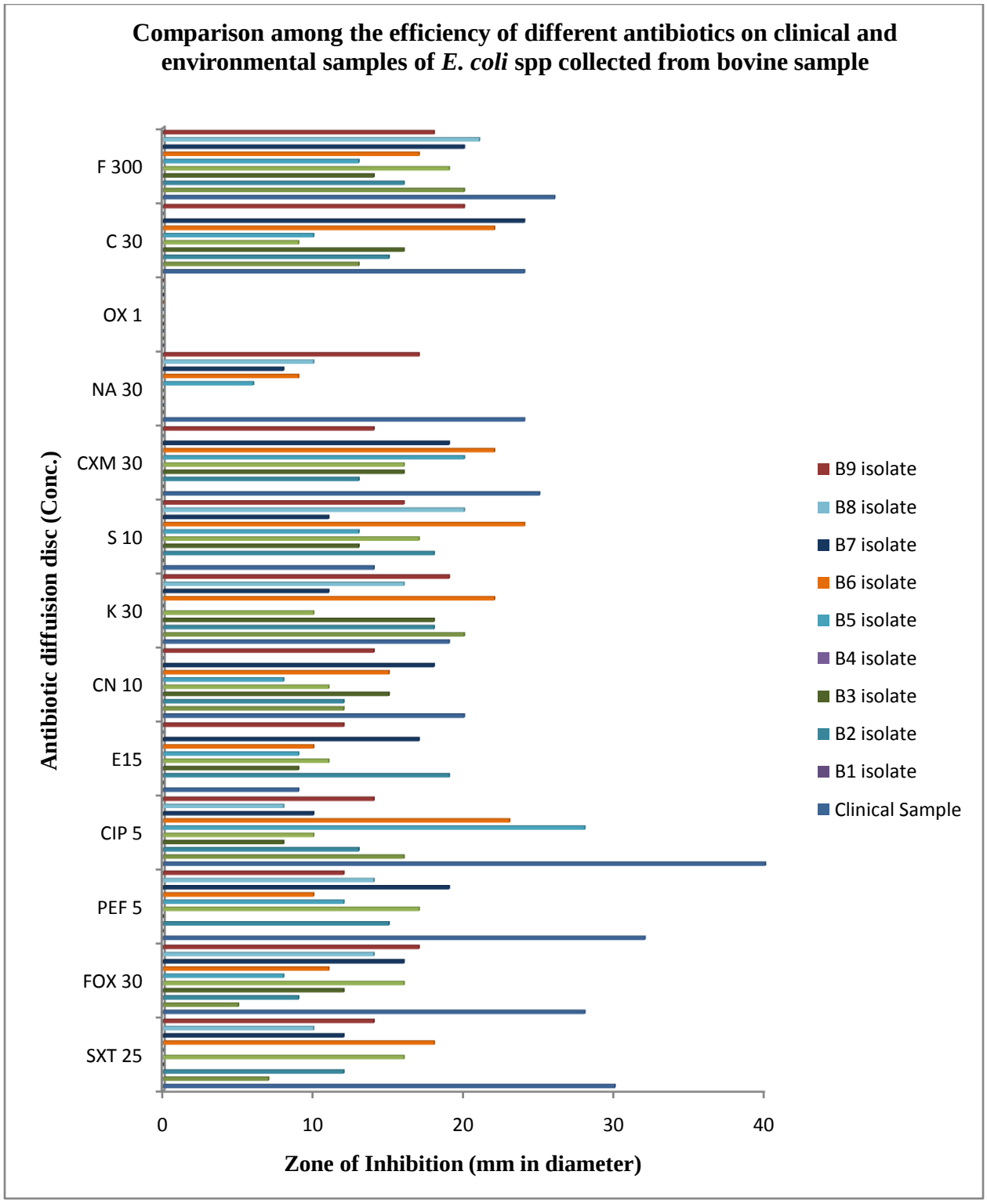


Figure 23: Comparison among the efficiency of different antibiotics on clinical and environmental samples *of E. coli* spp collected from bovine sample

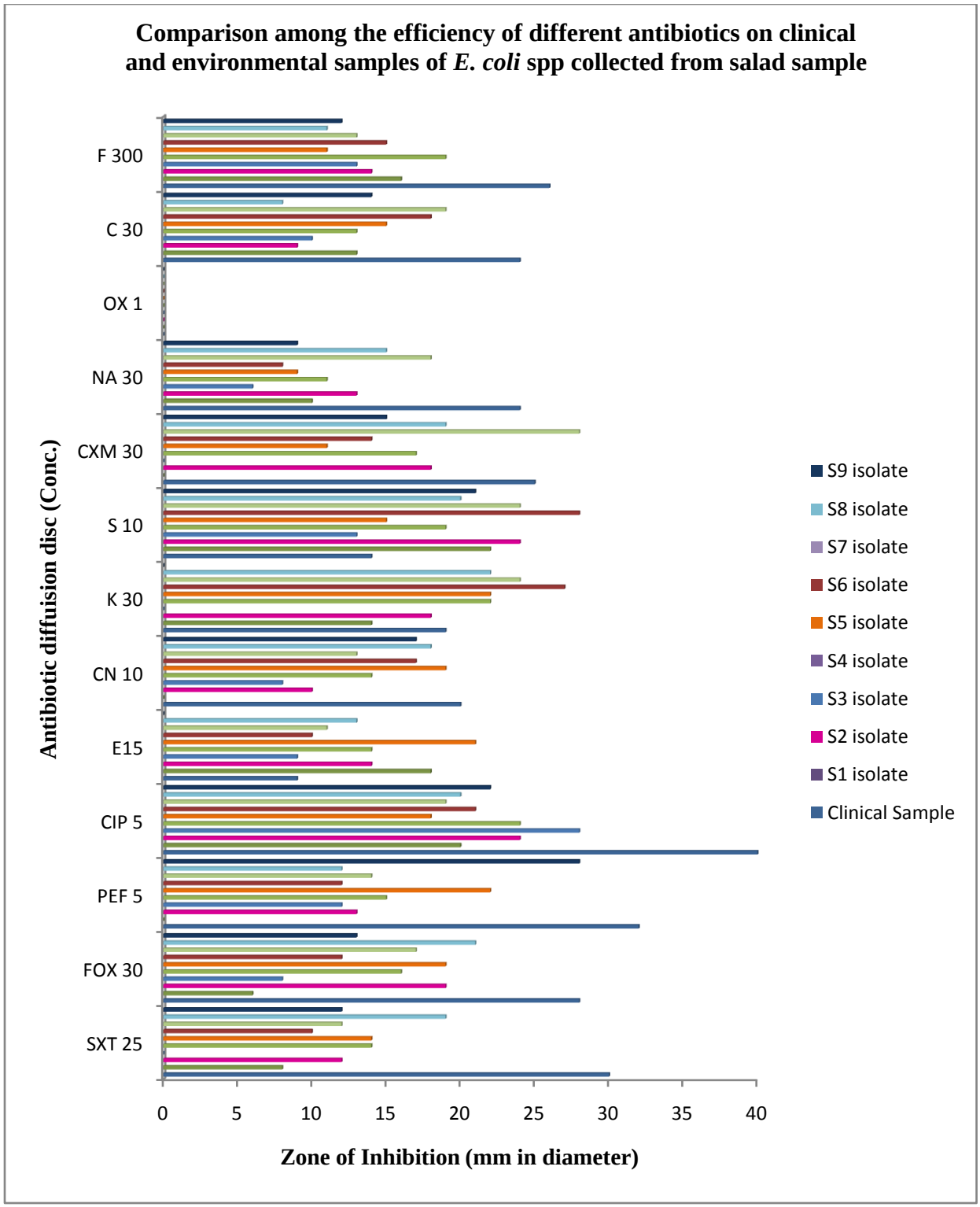


Figure 24: Comparison among the efficiency of different antibiotics on clinical and environmental samples *of E. coli* spp collected from salad sample

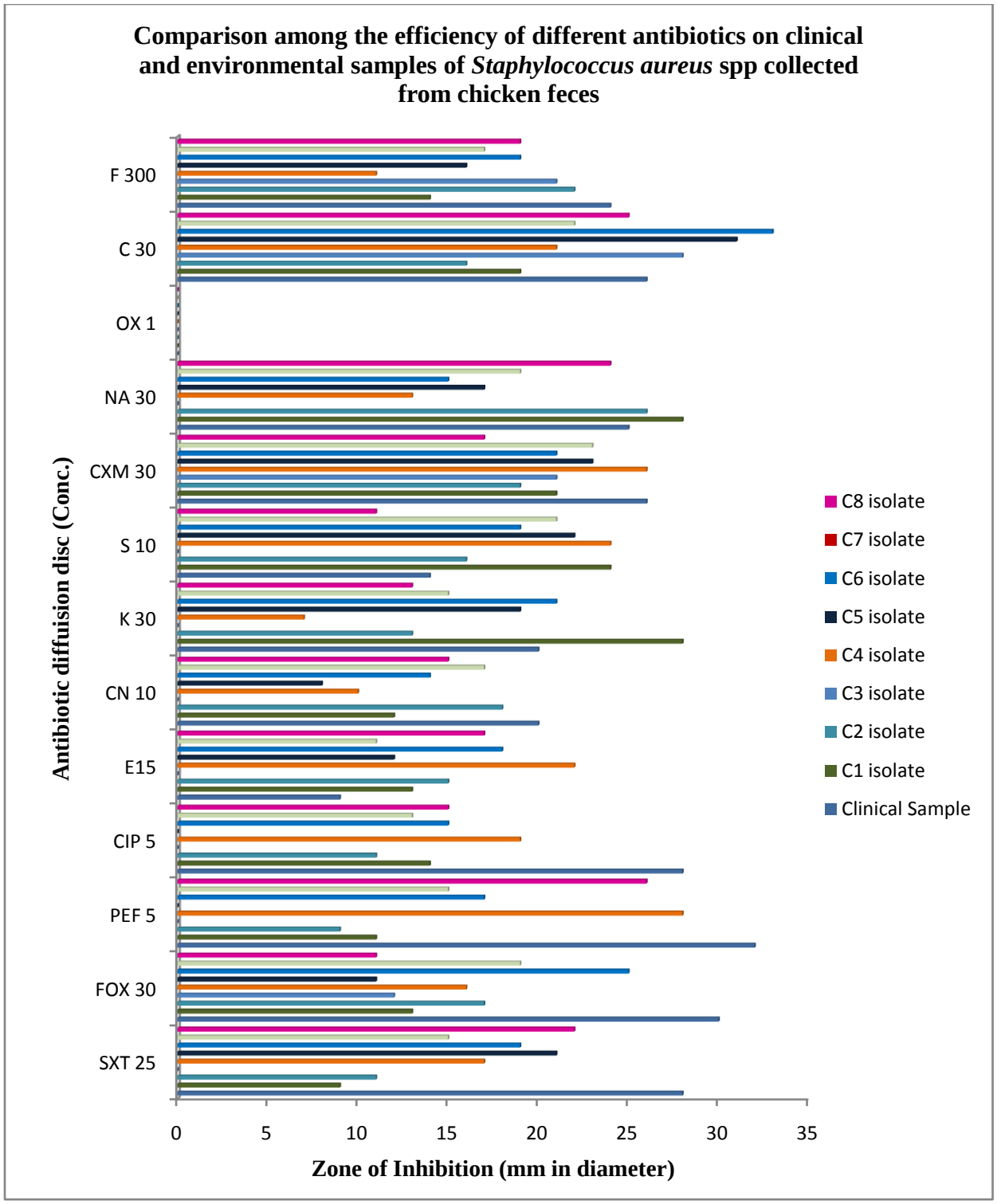


Figure 25: Comparison among the efficiency of different antibiotics on clinical and environmental samples of *Staphylococcus aureus* spp collected from chicken feces

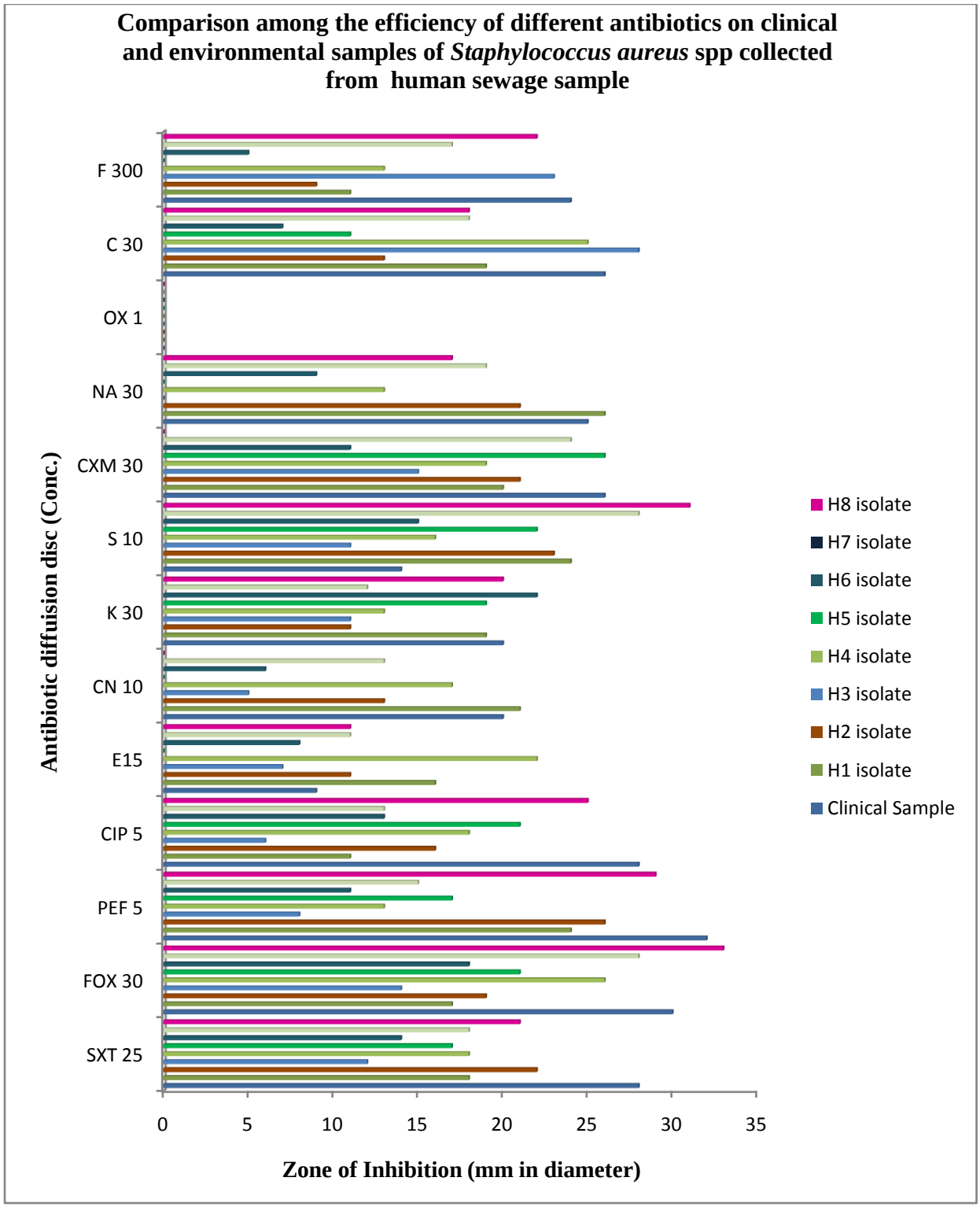


Figure 26: Comparison among the efficiency of different antibiotics on clinical and environmental samples of *Staphylococcus aureus* spp collected from human sewage sample

Chapter 4: Discussion

Chapter 4: Discussion

Antibiotic resistance is the ability of bacteria to endure the antimicrobial activity of antibiotics. It is now apparent that antibiotics that are used to alleviate an infection do not always work anymore. Antibiotic resistance is a global issue, and The US Centers for Disease Control and Prevention (CDC) considers antibiotic resistance one of their peak concerns [7].

One of the greatest discoveries of the 20th century is undoubtedly the finding of antibiotics. This fact is evident, but the genuine speculation is the rise of antibiotic resistance in hospitals, communities, and the environment is associated with their use. The surprising yet alarming new genetic capacities of microorganisms have facilitated from man's overuse of antibiotics to exploit every source of their resistance genes and every means of horizontal gene transmission to develop multi-resistant strains [8]. Such has been seen in this study, that the clinical strain of *Staphylococcus aureus* was significantly vulnerable to the antibiotics, yet the same strain isolated from the feces of chicken showed resistance to as many as eight different antibiotics. This is surely something to be concerned about.

Even prior to penicillin was introduced, resistant strains of bacteria had been detected. The selection pressure has been caused by the exercise of millions of antibiotics over the past 75 years. Ever since antibiotics were introduced to mankind, the abundant use of antibiotics has made almost all disease-causing bacteria resistant to the antibiotics that are commonly used to treat them [7].

Our environment contains numerous products that are man-made or triggered by human contamination, a large variety of examples can be petroleum chemicals, chemical solvents, the products and waste of industrial processes, heavy metals, garbage, and so on. Since the early development of the industrial revolution, humankind has dumped ever-growing amounts of organic and inorganic toxins into streams, rivers, seas, oceans, land, and as well as air. Before the discovery of antibiotics, arsenic, mercury, and iodine were used industrially and, as medicinal. Which under some circumstances, are still employed as such. The major bacterial solution to toxic challenges has taken the form of multivalent pumping systems that prevent intracellular accumulation of structurally diverse bactericidal and bacteriostatic substances [9].

Antimicrobial resistance is a global problem. Although antimicrobial agents have played a major role in reducing the threat from communicable diseases, but the widespread use of these synthetic agents has increasingly resulted in the development of microorganisms that are resistant. In recent years, resistance to these agents has been recognized as a major threat to public health. Emergence of multidrug resistance has limited the therapeutic options, so monitoring the resistance pattern has vital importance [10]. Resistance has increasingly become an even bigger problem in recent years due to the drastically slowed pace at which novel antibiotics are being discovered, while antibiotic use is rising rapidly [11]. This study presents the most important aspects of antibiotic resistance development through a longitudinal study, with the conclusion that it is time to take action. To achieve complete reimbursement of therapeutic applications of antibiotics, we need to gather more information on the rise of antibiotic resistance. Creative approaches to the discovery of novel antibiotics and their accelerated and controlled introduction to therapy are mandatory, given the recent situation of antimicrobial resistance [12].

The environmental strains of *Bacillus cereus, Staphylococcus aureus, Salmonella typhi, Shigella flexneri and Escherichia coli* are isolated from different potential environmental sources like salad sample, sewage water, chicken feces, bovine sample etc. Target environmental bacterial species were identified and used for the identification of antibiotic resistance pattern in compare to clinical strains obtained from ICDDR,B (International Center for Diarrheal Disease Research, Bangladesh). All strains were confirmed by means of specific biochemical tests and confirmed in selective media and maintained in nutrient ager medium.

B. cereus was isolated from two different environmental sample for this study, which are, human sewage sample and salad sample. Isolates from both samples showed much less resistance pattern than the other test organisms. *B. cereus* S3 collected from salad sample showed resistance to FOX 30 and *B. cereus* S6 showed resistance to PEF5, and F 300 (Figure 11). Whereas, the other isolates from salad sample showed no resistance to any of the test antibiotics. On the other hand, *B. cereus* H1 isolated from human sewage sample was observed to be resistant to PEF5 and *B. cereus* H2 was resistant to CIP5 and S10. No other *B. cereus* isolated from human sewage sample was resistant to any antibiotics (Figure 12).

In this study, two different isolates of *S. typhi* showed resistance to different antibiotics. For human sewage sample isolates, *S. typhi* H1 showed resistance to FOX 30, E15, and S10. Additionally, *S. typhi* H3 was resistant to SXT25, PEF5, E15, and C30. No other *S. typhi* isolates showed any resistance pattern (Figure 13). The isolates of *S. flexneri* collected from chicken feces showed very little resistance pattern. The isolate *S. flexneri* C3 was resistant to FOX30, and E15. No other *S. flexneri* isolates were observed to be resistant to any of the tested antibiotics (Figure 14).

E. coli was also isolated from two different sources for this study, bovine and salad sample. Isolates from both sample showed astounding resistance pattern. Among the isolates from bovine sample, *E. coli* B1 was resistant to PEF5, E15, and S10, E. *coli* B3 was resistant to SXT25, PEF5, and NA30. *E. coli* B5 was resistant to SXT25, and K30. *E. coli* B8 was observed to be resistant to four different antibiotics, which are E15, CN10, CXM30, and C30. All the other isolates from bovine sample showed resistance to none of the tested antibiotics (Figure 15). *E. coli* isolates from salad sample also showed great resistance pattern. *E. coli* S1 was resistant to PEF5, CN10, and CXM30, *E. coli* S3 was resistant to SXT25, and K30, and CXM30. *E. coli* S9 showed resistance to E15, and K 30. However, no other *E. coli* isolates from salad sample was resistant to any of the test antibiotics (Figure 16).

From chicken feces and human sewage sample, *S. aureus* was isolated. Isolates from both sample showed astonishing resistance pattern. Among the isolates from chicken feces, *S. aureus* C3 was resistant to as many as eight different antibiotics; they are SXT25, PEF5, CIP5, E15, CN10, K30, S10, and NA30 and *S. aureus* C5 was observed to be resistant to PEF5, and CIP5. Other *S. aureus* isolates from chicken feces showed resistance to none of the antibiotics (Figure 17). Among the isolates from human sewage sample, *S. aureus* H3 was resistant to NA30. *S. aureus* H5 showed resistance to four different antibiotics, which are E15, CN10, NA30, and F300. *S. aureus* H8 was observed to be resistant to CN10, and CXM30. Other *S. aureus* isolates collected from human sewage sample showed resistance to none of the test antibiotics (Figure 18). Result of this study shows that these organisms have been well exposed to the tested antimicrobials and they have developed mechanisms to avoid them.

Similar studies were done in various parts of the world to know the resistance pattern of different microorganisms. As example, it is well established that methicillin resistant *Staphylococcus aureus* (MRSA) has been a major challenge for the last 30 years, and in 1993, a study had examined *S. aureus* isolates from 9 different hospitals and found that 15% of isolates were resistant to methicillin [13]. Whereas, in this study *S. aureus* isolated from environmental sample (chicken feces) were found to be resistant to as many as eight different antibiotics, which are SXT25, PEF5, CIP5, E15, CN10, K30, S10, and NA30. This is a drastic change brought on by evolution.

Likewise, in Ireland, data that has been collected since 1996 had found high levels of resistance among *Salmonella* enterica serotype *Typhimurium*. Many isolates had been found to be resistant to at least 5 antibiotics, Ampicillin, Chloramphenicol, Streptomycin, Sulphonamide and tetracycline (ACSSuT). In this study *S. typhi* isolated from human sewage sample was observed to be resistant to three to four different antibiotics [14]. Among them are FOX30, E15, S10, SXT25, PEF5, and C30. This data only supports the fact that organisms are evolving in every possible ways and are gaining resistance to different and newer antibiotics. In this past decade, various key organizations, including the Infectious Diseases Society of America, the Centers for Disease Control and Prevention, and the World Health Organization (WHO), have made antibiotic resistance the focus of highly visible reports, conferences, and actions [15].

Genetic variability is essential for survival and antimicrobial agents will favor those organisms capable of resisting them. Microorganisms are either inherently resistant, that is, resistance determined by the basic nature of the organism or they can develop acquired resistance, which is the resistance that develops in a previously sensitive strain. Acquired resistance has generally been found to be derived from the use of antimicrobials. In addition, resistance problems are greatest in countries with highest use and in areas where use is concentrated, like intensive therapy units [9].

Antimicrobial resistance pattern monitoring will help us to review the current status of antimicrobial resistance locally, nationally and globally and helpful in minimizing the consequence of drug resistance, limit the emergence and spread of drug resistant pathogens. This has been a major endeavor of this study. Resistance to antibiotic is increasing and significant community health problems are at risk. An accelerated start up of developing new antibiotics and taking measures to conserve the existing microbial agents can be our way to alleviate the current problem. Also the widespread usage of antibiotics should be brought to a controlled manner along with the measures to help control the bacterial spread to slow the emergence and spread of resistant organisms.

Chapter 5: Conclusion

Chapter 5: Conclusion

Undoubtedly, we are living in the wonder years of modern medical sciences and enjoying a longer and healthier life. Antibiotics are one of the major reasons behind this, as it has been our vital source in fighting against various infectious diseases. Since the very beginning of antibiotics starting with the discovery of Penicillin, a diverse range of antibiotics have aided us persistently in our battle against the diseases.

It is alarming that despite all these advances in medical science and development of new antibiotics, the rate of morbidity and mortality due to antibiotic resistant pathogens are increasing. One of many reasons behind this is the widespread antibiotic usage, as it drives the pathogens to develop resistance. Now it is crucial to access the resistance pattern of pathogenic organisms to come up with a solution. This present study has been done in reflection to this fact, and the conclusion can be drawn that this comparative analysis will take us one step closer to resolving this global problem.

References

1. Woolhouse, M.E.J. 2002. Population biology of emerging and re-emerging pathogens. *Trends in Microbiology.* Vol. 10 No. 10.
2. Bonjar, G.H.S., Nik .A.K. and Aghighi. S. 2004. Antibacterial and antifungal survey in plants used indigenous herbal-medicine of south east regions of Iran. *Journal of Biological Sciences*.Vol.4 (3). pp. 405-412.
3. Abiramasundari, P., V. Priya, G.P .Jeyanthi. and Devi. S Gayathri. 2011. Evaluation of the Antibacterial activity of *Cocculus hirsutus*. *Hygeia. J. for Drugs and Med* .Vol.3 (2): 26-31.
4. Lambert, M. 2011. Molecular Biosafety - An update on alternatives to antibiotics - old and new strategies. *Applied Biosafety*: *ABSA*. Vol.16 (3).
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. 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.
7. Antibiotic resistance—the need for global solutions, LIDC. 2013.
8. Fischbach, M.A., and Walsh, C.T. 2010. Antibiotics For Emerging Pathogens. *Science.* Vol. 325. pp. 1089-1093.
9. Davies, J., and Davies, D. 2010. Origins and Evolution of Antibiotic Resistance. *Microbiol Mol Biol Rev*. Vol. 74 (3). pp. 417–433.
10. Abdullah, S., J. Gobilik and K. P. Chong. 2013. In Vitro Antimicrobial Activity of *Cynodon dactylon* (L.) Pers. (bermuda) against Selected Pathogens. *International Journal of Pharmacy and Pharmaceutical Sciences*. Vol. 4. (5). pp. 227-230.
11. Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. 2014.
12. Smith, R.D., and Coast, J. 2002. Antimicrobial resistance: a global response. *Bulletin of the World Health Organization*. Vol. 80. pp. 126-133.
13. Dála, M.M.T. 2001. A Strategy for Control of Antimicrobial Resistance in Ireland. Health and Children.

14. Nathan, C., and Cars, O. 2014. Antibiotic Resistance - Problems, Progress, and Prospects. *The New England Journal of Medicine*. Vol. 371. pp. 1761-1763.
15. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition, CLSI. 2012. Vol. 32 No. 2.

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^{o}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. Cetrimide agar (Merck, India)

Ingredients	Amount (g/L)
Pancreatic digest of gelatin	20.0
Magnesium chloride hexahydrate	1.4
Potassium sulfate anhydrous	10.0
Cetrimide	0.3
Agar-Agar	13.0

4. T_1N_1 soft agar

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

.

5. Tryptone soy broth, (Oxoid, England)

Ingredients	Amount (g/L)
Pancreatic digest of Casein	17.0
Papaic digest of soybean meal	3.0

Sodium chloride	5.0
Di-basic potassium phosphate	2.5
Glucose	2.5

6. MacConkey agar (Oxoid, England)

Ingredients	**Amount (g/L)**
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

7. 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

8. Peptone Water

Ingredients	**Amount (g/L)**
Peptone	10.0
Sodium chloride	5.0

9. MR-VP broth

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

10. 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

11. Eosine methylene blue agar (Oxoid, England)

Ingredients	**Amount (g/L)**
Peptone	10.0
Sucrose	5.0
Lactose	5.0
Di-potassium phosphate	2.0
Eosin Y	0.14
Methylene blue	0.065
Agar	13.50

12. Mannitol Salt agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	10.0
Manitol	10.0
Lab-lemco powder	1.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

13. Thiosulfate Citrate Bile Salts Sucrose agar (Difco, USA)

Ingredients	Amount (g/L)
Proteose peptone	10.0
Sodium thiosulfate	10.0
Sodium citrate	10.0
Yeast extract	5.0
Oxgall	8.0
Sucrose	20.0
Sodium chloride	10.0

Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.0

14. 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
Yeast extract	3.0

15. 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

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 Na_2HPO_4 and 2.0 g of KH_2PO_4 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 4^oC.

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^oC.

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^oC.

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,N^1,N^1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4^oC.

APPENDIX-III

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^{o}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