Serum Bactericidal Activity Against Multi-Drug Resistant *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*

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

Department of Mathematics and Natural Sciences

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BRAC University

June, 2022

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Declaration

It is hereby declared that-

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3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Acknowledgement

In the beginning, we hope to express our thankfulness to the Almighty Allah for blessing us with great opportunities to complete our thesis. We would also like to thank our families and friends for their unconditional support which helped us finish the thesis successfully.

After that, I want to voice my true-hearted gratitude to our supervisor Dr. M. Mahboob Hossain (Professor, Department of Mathematics and Natural Sciences, BRAC University) and Co-supervisor (Akash Ahmed, Lecturer, Department of Mathematics and Natural Sciences, BRAC University) for their dedicated, insightful and inspiring supervision throughout the thesis journey. It was an absolute blessing to work under them.

Our special thanks go to our departmental head Professor A F M Yusuf Haider (Professor & Chairperson, Department of Mathematics and Natural Sciences, BRAC University) for providing us with the scope to work on research under their constant guidance.

At last, we are thankful to all the individuals who influenced and encouraged us to complete our thesis successfully as this was a significant opportunity for us to acquire basic skills in the way of our career which will certainly help us in our near future.

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List of Abbreviation and Codes:

E. coli - *Escherichia coli*

K. pneumoniae - *Klebsiella pneumoniae*

S. aureus - *Staphylococcus aureus*

P. aeruginosa - *Pseudomonas aeruginosa*

EGTA - Ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid

MHA – Mueller Hinton Agar

EMB – Eosin Methylene Blue

MSA – Mannitol Salt Agar

Hib - Haemophilus influenzae type b

MDR – Multidrug resistant

ABR – Antibiotic resistance

Abstract

Antibiotic resistance has become a matter of utmost concern. Many disease-causing pathogens are becoming resistant to multiple antibiotics, referred to as multi-drug resistant (MDR) bacteria. The purpose of this study is to know the immunity against MDR bacteria among normal healthy individuals. Bacterial samples were collected from tertiary care hospitals. Gram staining followed by biochemical tests were performed to confirm the identity of the four different organisms- *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. After identification, antibiogram was performed to confirm that they are MDR bacteria. From normal individuals' blood was collected and centrifuged at 4000 rpm for 10 min to obtain serum. All serum samples were incubated with four different bacterial suspensions. All sera samples were heated at 56℃ for 30 minutes to inactivate them and then incubated with bacterial suspension. Lastly, bacterial suspension was incubated with sera to which ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) had been added. This was done to inhibit the classical pathway of the complement system. For *Escherichia coli*, *Pseudomonas aeruginosa,* *Klebsiella pneumoniae* and *Staphylococcus aureus*, **67.82%, 99.56%, 48.33%** and **93.38%** highest inhibition was observed respectively. No inhibition of bacterial growth was observed when treated with inactivated sera. Incubation with EGTA-added sera showed uncontrolled bacterial growth which suggests that the observed bactericidal effect of sera was due to the classical pathway and no other pathway of the complement system was involved.

Keywords:

Multidrug resistant, bacteria, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa,* blood, serum, incubation, inhibition, ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA).

Chapter 1

Introduction

Introduction

1.1 Background

Globally, with each passing year, the fatality of antibiotic resistance is just rising with a decline in the development of new antibiotics, which scenario is just posing a risk in terms of economic burden especially in developing countries. Bangladesh falls into the category of "Developing Country", the scenario is no exception. The factors contributing to this fatality include over-prescription of antibiotics, not completing the entire antibiotic course, poor infection control in the healthcare system, poor maintenance of hygiene in sanitation, non-human antibiotic use, inadequate surveillance, repeated infections and absence of the discovery of new drugs. A large study by Global Antibiotic Research and Development Partnership focused on newborn babies clinically diagnosed with sepsis and concluded that antibiotic resistance is contributing to neonatal sepsis causing their death (GARDP, 2022). A report on global surveillance of antimicrobial resistance by WHO in 2014 stated that significant gaps have been prevailing in surveillance along with a lack of standards for methodology, data sharing and coordination leading to the increase of multi-drug resistant bacteria (WHO, 2014).

Bangladesh bears a high degree of ABR which poses both a regional and global threat. In 2003, a study performed in Chittagong concluded that typhoid patients were found to show resistance to both first and second line (ciprofloxacin) therapy (Asna et al., 2003). Multiple studies revealed that all these kinds of therapeutic failures in Bangladesh mostly resulted from a practice of self-medication among patients lacking any prior knowledge, irrational antibiotic prescribing by physicians and indiscriminate use of antibiotics in livestock (Biswas et al., 2014).

1.2 Serum Bactericidal Assay Activity

The SBA test is the method of choice for determining the classical complement-mediated functional activity of both infection and vaccine induced antibodies. Since the late 1800s, this phenomenon has been frequently seen and investigated, and it has been proven to be complement mediated. Serial dilutions of sera are incubated with target bacterial strains which are then plated on respective selective media followed by overnight incubation in a standard SBA experiment. Previously, many studies were carried out on SBA as the bactericidal effect of normal human serum plays an important role in host defense through their classical complement mediated pathway against bacterial infection. It helps to gain an understanding of the bacteria and also the

host is affected by it and also proved to work as an investigational tool for comparing the respective efficacy of antibiotics administered in combination or alone (Klastersky, 1980). One of the studies on SBA showed that it has been observed that the infected serum mediated killing differs from organism to organism (Shahriar et al., 2010).

1.3 Classical Complement Mediated Pathway

In 1896, Jules Bordet discovered a complement mediated pathway that acts as a heat-labile component of normal plasma causing the death of bacteria. The complement system refers to a series of >20 proteins that are found circulating in the blood and tissue fluids. Most of the proteins normally stay in an inactive state but when they recognize any of the molecular components of microorganisms, they become sequentially activated in an enzyme cascade. The activation of one protein enzymatically cleaves and activates the next protein in the cascade. The classical complement pathway is one of three pathways that activates the complement system and is part of the immune system. The classical complement pathway is initiated by the binding of antigen-antibody complexes with the antibody isotypes IgG and IgM. So, the binding of C1 protein with the Fc component of the antibody initiates this pathway. After binding they start to recruit complement proteins. They first recruit C4 protein which eventually gets cleaved into two components-C4a and C4b. The recruited C2 protein which also gets cleaved into C2a and C2b. C4a and C2a diffuse away while C4b and C2b get deposited on a pathogen surface which forms a complex together known as C3 convertase. Then, the C3 convertase cleaves into C3a and C3b which is the point at which all complement activation cascades converge. Again, C3a gets diffused and C4b, C3b and C2b together form C5 convertase. C5 convertase will recruit C5 protein and will cleave it to C5a and C5b. C5b will be deposited on the surface of the bacteria. C5b then attracts other complement proteins C6, C7 and C8 and will recruit them. All of them get deposited and recruit C9. It causes cell lysis by forming pores. The formation of pores results in disruption of the osmotic balance of cells which ultimately causes their death. And this phenomenon is known as Membrane attack complex (MAC) (Dunkelberger and Song, 2010).

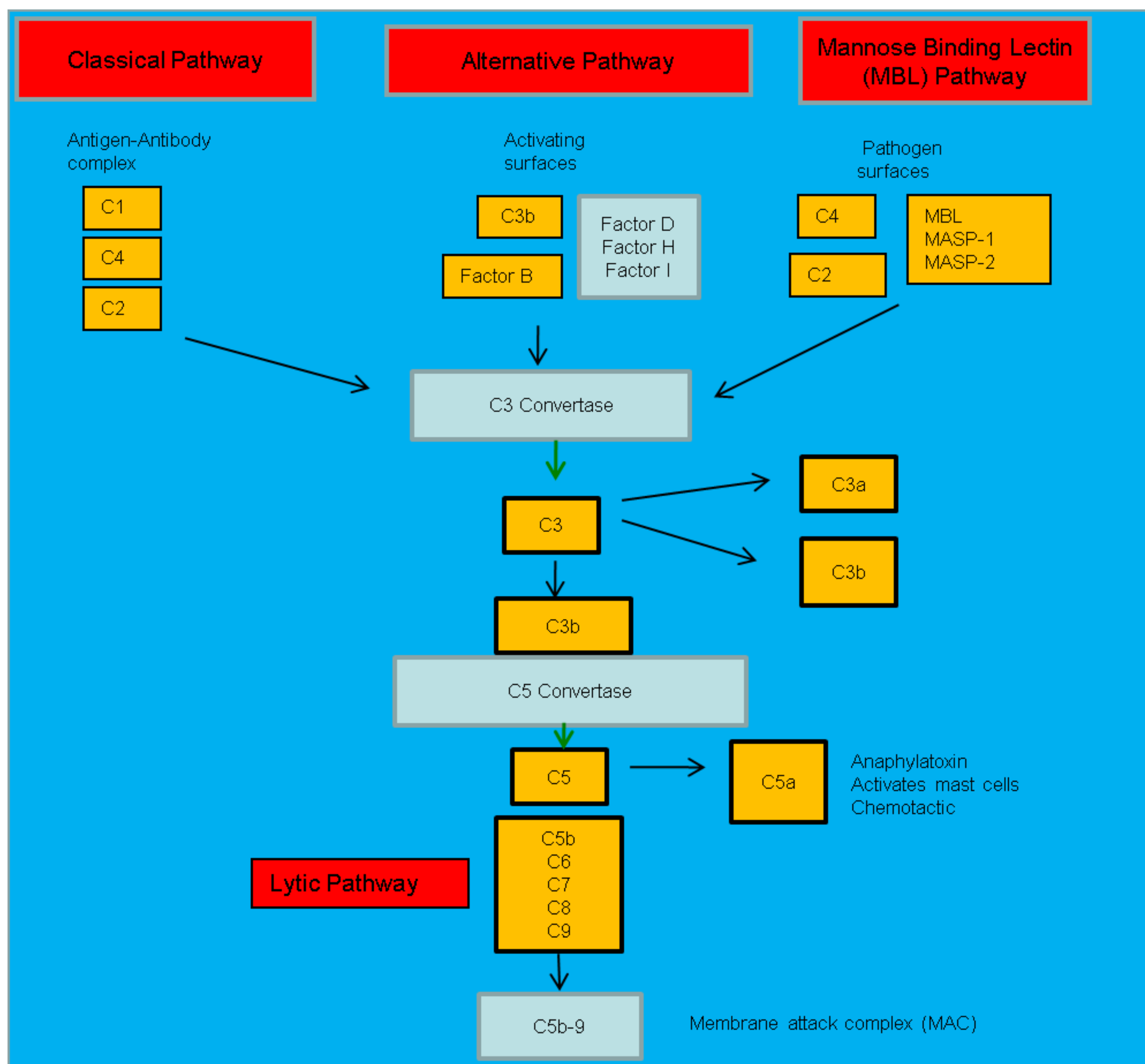

Figure 1.3.1: Complement mediated pathway.

1.4 Current study

Considering the present scenario of Bangladesh, the current study was conducted to calculate the predominance of the four MDR organisms, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae* through serum bactericidal activity assay. So, the purpose of this study was to gain an understanding of the exposure of Bangladeshi people to MDR bacteria by determining the prevalence of immunoglobulin against them.

Bacterial samples were collected from tertiary care hospitals. Gram staining followed by biochemical tests were performed to confirm the identity of the four different organisms- *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

After identification, antibiogram was performed to confirm that they are MDR bacteria. Blood was collected from normal individuals and centrifuged at 4000 rpm for 10 min to obtain serum. Our study was conducted following the standard SBA experiment procedure where the serial dilutions of sera were incubated (varied incubation times was maintained) with target organisms which were then plated on respective selective media followed by overnight incubation. Lastly, bacterial suspension was incubated with sera to which ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) had been added. This was done to inhibit the classical pathway of the complement system. After that, the count of the surviving bacterial colony forming units (CFU) at each serum dilution was taken and the inhibition percentage was determined using the formula of inhibition.

For *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, **67.82%, 99.56%, 48.33%** and **93.38%** highest inhibition was observed at varied incubation times, respectively. No inhibition of bacterial growth was observed when treated with inactivated sera. Incubation with EGTA-added sera showed uncontrolled bacterial growth which meant that the observed bactericidal effect of sera was due to the classical pathway and no other pathway of the complement system was involved.

Literature Search:

The serum is widely known to possess bactericidal activity against a number of susceptible organisms particularly gram-negative genus. Complement mediated bactericidal activity of serum has been studied since the late 1800s (Inoue et al., 1968). Earlier, our body has been colonized by many commensal bacteria in the intestinal tract. This colonization ensured small quantities of antibodies which are IgM and IgG, be present in the blood and tissues of both humans and animals, directed against the surface antigens of many types of gram-negative bacteria like *Escherichia coli* (Mason & Richardson, 1981, Šterzl, 1962, Cohen & Norins, 1966, Michael & Rosen, 1963).

In this study, patients infected with *Salmonella typhi* are selected to observe their serum bactericidal activity against *Salmonella typhi* and also the important role played by complement pathways (classical and alternative) are also examined by inactivating both pathways of *S. typhi* infected human sera. As per Taylor and Kroll (Taylor & Kroll, 1983), the serum bactericidal assay for all nine normal human sera and *S. typhi* infected human sera were carried out. Here, the mean values of the log10CFUs of *S. typhi* observed after serum bactericidal assay at various incubation time in per ml of bacterial cell suspension treated with each of nine *S. typhi* infected human sera, nine normal human sera, and nine heat-treated *S. typhi* infected human sera were calculated. The mean growth of *S. typhi* in the presence of infected human sera showed decline than the positive control after different incubation time. So, this study concluded that after 90 minutes of incubation time, *S. typhi* infected serum mediated killing showed maximum activity (Shahriar et al., 2011).

Determination of bactericidal activity in serum provides much needed information on the host, the bacteria and the antibiotics. Through clinical studies, it has become successful in proving its value by predicting the outcome of septicemia and other infections. Here, cancer populations were screened out of gram-negative septicemias who were also found not to be neutropenic. The correlation was established between their clinical outcome and the level of the bactericidal dilution of the serum where a plateau was observed indicating that increasing the antibacterial activity in the serum is not useful after reaching a peak bactericidal dilution of 1:8 or 1:16. One of the two phenomena might have taken place; failure of antibiotics in reaching the site of infection or the host defense being severely compromised. This phenomenon was described by

Wade and Schimpff (Wade & Schimpff, 1982) recently in neutropenic leukemic patients where rate of response was found almost the same even when double or triple antibiotics were used. They made use of three antibiotics- aminoglycoside, carbenicillin and cephalosporin. Also, this study focused on comparative studies of two antibiotics named gentamicin and amikacin that were made against gram negative bacilli by comparing the serum bactericidal activity in volunteers. It concluded that there is no difference in the efficacy of these two drugs (Klastersky, 1983).

The bactericidal activity in serum has shown its usefulness in evaluating antibiotic synergism. At last, this work focused on evaluating synergism, using 20 strains of Klebsiella sp. correlation was established between the serum bactericidal activity in 10 subjects after receiving cefazolin, amikacin and a combination of these two drugs with the results of in vitro tests for synergism. Also, a comparison was made between the serum bactericidal activity in patients infected with strains against which the antibiotic used were synergistic in vitro with that in patients infected with strains where no synergism could be found (Klastersky, 1983). A review of six studies found in the literature concluded that the synergistic combinations resulted in better activity than the non-synergistic ones (Klastersky, 1980).

Chapter 2

Materials and Methods

2.1 Location of study

This study was carried out in the Microbiology Laboratory, Department of Mathematics and Natural Sciences, BRAC University.

2.2 Collection of samples

Four MDR bacteria (Shown in table 1.1) were collected from the National Institute of Diseases of the Chest and Hospital (NIDCH), situated at Mohakhali, Dhaka. Taking them into the Microbiology Research Laboratory of the department of Mathematics and Natural Sciences of BRAC University, they were subjected to experimental works maintaining proper safety rules which are serially stated below.

Table 2.2.1: Name of collected organisms and hospital

Name of Organisms	Name of the Hospital
Escherichia coli	National Institute of Diseases of the Chest and Hospital
Klebsiella pneumoniae	National Institute of Diseases of the Chest and Hospital
Pseudomonas aeruginosa	National Institute of Diseases of the Chest and Hospital
Staphylococcus aureus	National Institute of Diseases of the Chest and Hospital

2.3 Antibiotic Susceptibility Test

Four of the organisms were selected for a detailed antibiogram study by Kirby-Bauer disc diffusion susceptibility testing, using the commercially available standard antibiotic disks as stated in the table 3.2.1, 3.2.2, 3.2.3 and 3.2.4. Upon incubation for 18-24 hours at 37°C the MHA plates were observed and the resistance profiles were determined following CLSI standards (2021).

2.4 Biochemical Tests

Before starting any kind of experimental work, it was very important to confirm the identity of the collected organisms for which the following biochemical tests were performed by us for four of the organisms.

2.4.1 Methyl Red

- Four broths containing glucose and phosphate buffer were taken in which collected *E. coli, K. pneumoniae, S. aureus* and *P. aeruginosa* were inoculated, followed by incubation at 37℃ for 24 hours.
- After incubation, four drops of methyl red were added.
- In a positive reaction, the color of the medium turned red. The ellow color indicated negative results.

2.4.2 Voges Proskauer

- Four glucose phosphate broths were taken in which collected organisms were inoculated, followed by incubation at 37℃ for 48 hours.
- After incubation, ten drops of alpha-naphthol (Barritt's A) were added first and then ten drops of potassium hydroxide (Barritt's B) were added.
- In a positive reaction, the color of the medium turned red. The yellow color indicated negative results.

2.4.3 Citrate Utilization Test

- Desired organisms were inoculated into a slope of Simmon's citrate agar, followed by incubation at 37℃ for 24 hours.
- The change in color was observed after incubation.
- In a positive reaction, the color of the medium turned blue. No change in color indicated negative results for citrate utilization.

2.4.4 Catalase Test

- A small amount of the desired organism was collected using a sterile inoculating loop and was placed on a microscopic slide.
- After that, a drop of 3% H_2O_2 was added to it using a dropper.

- The formation of bubbles indicated positive results i.e., the organism can utilize H_2O_2.

2.5 Gram Staining

Gram staining is considered one of the most crucial staining techniques for the identification of bacteria on the basis of their cell wall constituents classifying them into two groups - gram positive (stains violet) and gram-negative (stains red) bacteria. For this reason, to reach a more confirmed conclusion gram-staining was done for four of the collected organisms following standard procedure.

2.6 Subculture of *E. coli* on MacConkey Agar

MacConkey agar is a selective and differential media that is used for the isolation and differentiation of the members of the family Enterobacteriaceae. It not only selects for Gram-negative organisms by inhibiting Gram-positive organisms but also differentiates the Gram-negative organisms by lactose fermentation.

E. coli being one of the members of Enterobacteriaceae family, MacConkey is their selective media for which it was chosen for subculture. Under laminar hood, by picking an isolated colony from the collected stock, it was transferred to a fresh new MacConkey agar plate by streaking in a zigzag method. It was then incubated overnight at 37℃. After incubation, many colonies appeared that were red because the production of acid took place from lactose that reduced the pH below 6.8.

2.7 Subculture of *K. pneumoniae* on EMB Agar

EMB is a differential media that is used for the isolation and differentiation of gram-negative enteric bacteria. It not only selects for Gram-negative organisms by inhibiting Gram-positive organisms but also differentiates the Gram-negative organisms by lactose fermentation by providing a color indicator.

EMB agar is a selective media for *K. pneumoniae* for which it was chosen for subculture. Under laminar hood, by picking an isolated colony from the collected stock, it was transferred to a fresh new EMB agar plate by streaking in a zigzag method. It was then incubated overnight at 37℃.

After incubation, many colonies appeared that were pink in color because the production of acid took place from lactose that reduces the pH.

2.8 Subculture of *S. aureus* on MSA

MSA is used as a selective and differential medium for the isolation and identification of *S. aureus*. The MSA agar contains beef extract and protease peptone that act as the source of nutrients, growth factors, vitamins, minerals, and amino acids required for the growth of microorganisms. It contains a 7.5% concentration of sodium chloride that inhibit the growth of bacterial organisms other than *S. aureus*.

As MSA agar is a selective media for S. aureus so it was chosen for subculture. Under laminar hood, by picking an isolated colony from the collected stock, it was transferred to a fresh new MSA agar plate by streaking in a zigzag method. It was then incubated overnight at 37℃. After incubation, many colonies appeared that were yellow in color because they ferment mannitol to produce yellow colonies.

2.9 Subculture of *P. aeruginosa* on Cetrimide Agar

Cetrimide agar is primarily used for selective isolation and identification of *P. aeruginosa.* The cetrimide agar contains gelatin peptone that provides necessary nutrients for growth, glycerol acts as the carbon source and Sodium chloride maintains osmotic equilibrium in the medium. It contains a toxic substance named cetrimide which inhibits the growth of many bacteria.

As cetrimide agar is a selective media for *P. aeruginosa* so it was chosen for subculture. Under laminar hood, by picking an isolated colony from the collected stock, it was transferred to a fresh new MSA agar plate by streaking in a zigzag method. It was then incubated overnight at 37℃. After incubation, many colonies appeared that were white or colorless.

2.10 Experiment Procedure

2.10.1 Before experiment

- Blood was collected from 20 volunteers of our Microbiology Research Laboratory and centrifuged at 4000 rpm for 10 minutes to obtain serum.
- Then the sera were separated carefully into microcentrifuge tubes and were stored at 20℃.

- The serum bactericidal assay for all sera samples was carried out according to Thomassen and Demko (Thomassen, 1981)
- Bacterial suspension of 1.5×10^{-8} colony forming units (CFU/ml) was prepared for four of the organisms compared with 0.5 McFarland turbidity standard.
- Each of the sera samples was treated with four of the collected organisms.

2.10.2 During experiment

The experiment was run in three parts as follows -

- Without Serum: Tenfold serial dilutions were done for up to 10^{-6}.
- With Serum: A total volume of 200 microliters was maintained in a microcentrifuge tube.

i) 10 microliters bacterial suspension, 100 microliters serum, 90 microliters NaCl.

They were incubated for 30 minutes and 60 minutes at room temperature. After incubation they were diluted tenfold up to 10^{-3}.

- With Mg-EGTA: A total volume of 200 microliters was maintained in a microcentrifuge tube.

i) 10 microliters bacterial suspension, 40 microliters EGTA, 40 microliters $MgCl_2$, 10 microliters 0.9% NaCl and 100 microliters serum to bring the final concentration of both EGTA and $MgCl_2$ 10mM.

They were incubated for 30 minutes and 60 minutes at room temperature. After incubation they were diluted tenfold up to 10^{-3}. After that, 100 microliters of solution were transferred to petri-plates and were incubated at 37℃ overnight.

2.10.3 After experiment

After incubation, the colony counts were taken from each plate and following the formula of inhibition the percentage of inhibition was calculated.

2.11 Formula for calculation of the percentage of inhibition

$$\frac{(\text{No. of colonies without serum} \times \text{Dilution Factor} - \text{No. of colonies with serum} \times \text{Dilution Factor}) \times 100}{\text{No. of colonies without serum} \times \text{Dilution Factor}}$$

Chapter 3

Results

3.1 Biochemical Test Results

For each of the four organisms, the results for the methyl red test, Voges Proskauer test, citrate utilization test, catalase test, gram staining and morphology observed under a microscope helped us to reach a confirmed conclusion about their identity which is shown in table 3.1.1.

Table 3.1.1: Biochemical tests for the identification of bacteria

Name of organisms	Methyl Red Test	Voges – Proskauer Test	Citrate Utilization	Catalase Test	Gram Staining	Morphology
Escherichia Coli	+	–	–	+	–	Rod
Klebsiella pneumoniae	–	–	+	+	–	Rod
Staphylococcus aureus	+	+	+	+	+	Coccus
Pseudomonas aeruginosa	–	–	+	+	–	Rod

3.2 Antibiotic Susceptibility Profile

The results obtained from the antibiogram of the four bacteria confirmed the multidrug resistance of all the organisms.

Table 3.2.1: Antibiotic susceptibility test results for *Escherichia coli*

Name of the Antibiotics	Zone of Inhibition (mm)	Interpretation
Amikacin	25	Sensitive
Tetracycline	23	Sensitive
Doxycycline	17	Sensitive
Imipenem	13	Resistant
Piperacillin	0	Resistant
Azithromycin	9	Resistant
Amoxicillin	0	Resistant
Erythromycin	0	Resistant
Meropenem	9	Resistant
Norfloxacin	0	Resistant
Moxifloxacin	0	Resistant

Table 3.2.2: Antibiotic susceptibility test results for *Klebsiella pneumoniae*

Name of Antibiotics	Zone of Inhibition (mm)	Interpretation
Tetracycline	19	Sensitive
Doxycycline	14	Sensitive
Amikacin	0	Resistant
Imipenem	14	Resistant
Piperacillin	0	Resistant
Azithromycin	0	Resistant
Amoxicillin	0	Resistant
Erythromycin	0	Resistant
Meropenem	0	Resistant
Norfloxacin	0	Resistant

Table 3.2.3: Antibiotic susceptibility test results for *Pseudomonas aeruginosa*

Name of Antibiotics	Zone of Inhibition (mm)	Interpretation
Imipenem	27	Sensitive
Piperacillin	24	Sensitive
Meropenem	42	Sensitive
Amikacin	5	Resistant
Tetracycline	7	Resistant
Moxifloxacin	6	Resistant
Azithromycin	0	Resistant
Amoxicillin	0	Resistant
Erythromycin	0	Resistant
Doxycycline	0	Resistant
Norfloxacin	12	Resistant

Table 3.2.3: Antibiotic susceptibility test results for *Staphylococcus aureus*

Name of Antibiotics	Zone of Inhibition (mm)	Interpretation
Doxycycline	31	Sensitive
Imipenem	53	Sensitive
Amoxicillin	37	Sensitive
Amikacin	30	Sensitive
Azithromycin	28	Sensitive
Tetracycline	10	Resistant
Cefepime	18	Resistant
Erythromycin	13	Resistant
Kanamycin	13	Resistant
Nitrofurantoin	10	Resistant

The result of this study denotes the bactericidal activity of blood serum collected from patients with medical history against four different multidrug resistant bacteria which are *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae*. The bactericidal activity was measured in comparison with the bacterial suspension. For all the organisms, nutrient agar medium and also each specific agar medium plate was used by the spread plate method. The results from this study depict different percentages of inhibition for different organisms at different incubation times. However, for the results each dilution was plated twice and then the average of colonies count was taken for calculations. With the variation of incubation time, the counts of colonies did not vary by a significant amount so it was not repeated much further.

The results for each organism are discussed below in the tables and some significant pictures of the experiments are also added for better understanding.

3.3 ***Staphylococcus aureus***

It is a gram-positive bacterium which is one of the most well-known bacteria to spread disease in humans. Other than soft tissue infections, *S. aureus* can cause serious infections such as bloodstream infections, pneumonia, or bone and joint infections. In previous studies, gram positive bacteria showed more bactericidal activity than gram-negative ones (Lorian & Atkinson, 1978). In our results below the same phenomena can be noticed.

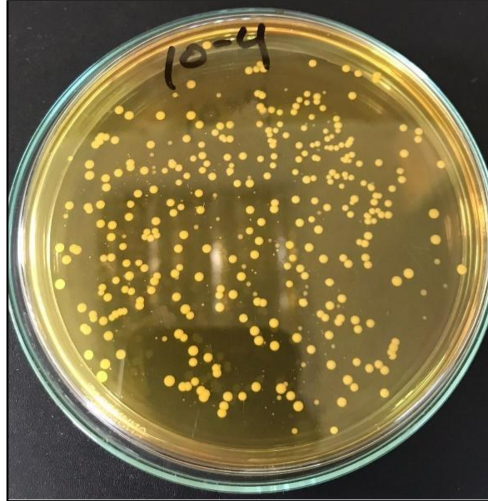

Figure 3.3.1: Growth of *Staphylococcus aureus* after incubation without serum at 10^{-4} dilution on MSA media.

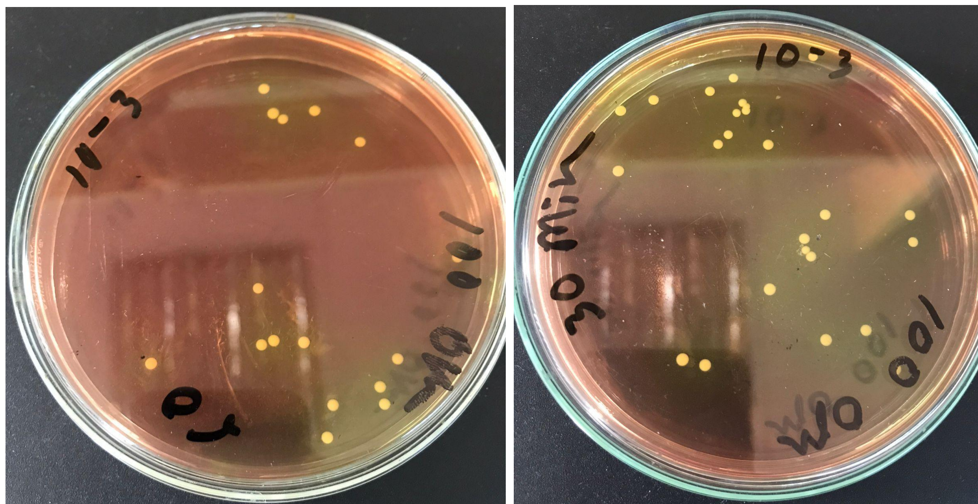

Figure 3.3.2: Noticeable inhibition in growth of *Staphylococcus aureus* after incubation with serum at 10^{-3} dilution at 30 and 60 minutes.

We can observe in the above images that increasing the incubation period slows the growth, but not by a considerable amount.

Table 3.3.1: Effect of serum on the inhibition of growth of *Staphylococcus aureus*

Date	Serum Initials	Incubation Time (Minute)	CFU without serum	CFU with Serum at Different dilution	CFU with Mg-EGTA	Inhibition % with Serum	Inhibition % with Serum & MG-EGTA
11.11.21	MH	60	10^{-4}=55 colonies CFU=5500000	10^{-2} = 267 colonies CFU=5340000	10^{3}= 195 colonies	2.9%	0%
11.11.21	FZ	60	10^{-4}=55 colonies CFU=5500000	10^{-2} = 203 colonies CFU=4060000	10^{-3}= 175 colonies	26.18%	0%
24.02.22	TA	60	10^{-4}=287 colonies CFU=28700000	10^{-3}=95 colonies CFU=19000000	10^{-3}= 211 colonies	33.79%	0%
24.02.22	AM	60	10^{-4}=287 colonies CFU=28700000	10^{-3}=75 colonies CFU=15000000	10^{-3}= 235 colonies	47.74%	0%
24.02.22	AS	60	10^{-4}=287 colonies CFU=28700000	10^{3}=60 colonies CFU=12000000	10^{-3}= 288 colonies	58.18 %	0%
24.02.22	SD	60	10^{-4}=287 colonies CFU=28700000	10^{-3}=90 colonies CFU=18000000	10^{-3}= 267 colonies	37.27%	0%
04.01.22	OM	60	10^{-4}=260 colonies CFU=26000000	10^{-2}=86 colonies CFU=1720000	10^{-2}=123 colonies	93.38%	90.53%

Date	**Serum Initials**	**Incubation Time (Minute)**	**CFU without serum**	**CFU with Serum at Different dilution**	**CFU with Mg-EGTA**	**Inhibition % with Serum**	**Inhibition % with Serum & MG-EGTA**
04.01.22	OM	30	10^{-4}=260 colonies CFU=26000000	10^{-2}=105 colonies CFU=2100000		91.92%	
22.11.21	AD	60	10^{-4}=189 colonies CFU=18900000	10^{-3}=29 colonies CFU=5800000	10^{3}= 55 colonies	69.31%	41.79%
22.11.21	SH	60	10^{-4}=189 colonies CFU=18900000	10^{-3}=17 colonies CFU=3400000	10^{-3}=60 colonies	82.01%	36.65%
22.11.21	SH	30	10^{-4}=189 colonies CFU=18900000	10^{-3}=65 colonies CFU=13000000		31.21%	
22.11.21	IF	60	10^{-4}=189 colonies CFU=18900000	10^{-3}=29 colonies CFU=5800000	10^{-3}=63 colonies	69.31%	33.33%
22.11.21	IF	30	10^{-4}=189 colonies CFU=18900000	10^{-3}=32 colonies CFU=6400000		66.14%	

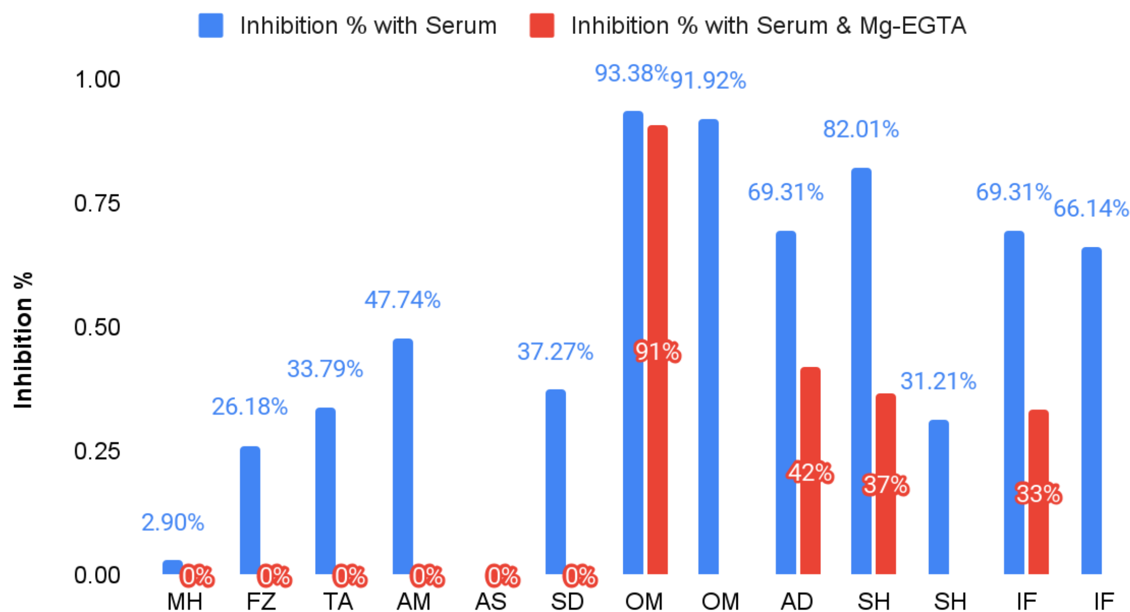

Figure 3.3.3: The inhibition of growth of *S. aureus* with serum and serum + Mg-EGTA respectively

The inhibition percentage ranged from lowest at 2% to the highest at about 93%, as seen in the figure 3.3.3. The inhibition differs from one individual to the next. The incubation time, on the other hand, has a negligible influence on the inhibition rate. The names that appear twice in the graph are the result of two separate incubation times. The gradients, however, are all in the same area on the graph. The serum containing Mg-EGTA exhibited negligible or no inhibition in some individuals, suggesting that the Mg-EGTA had no impact.

3.4 ***Klebsiella pneumoniae***

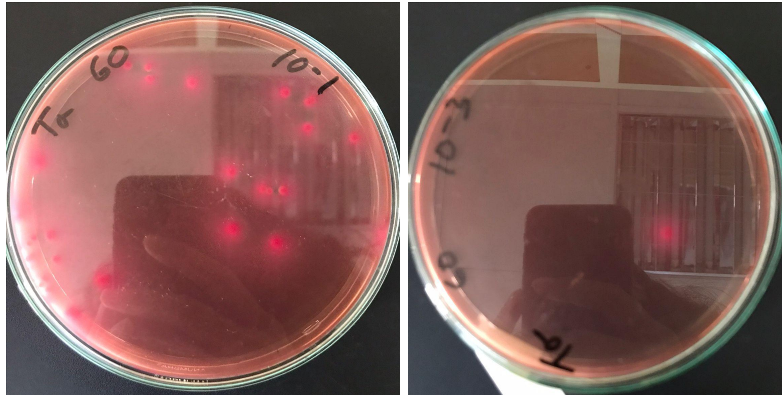

Figure 3.4.1: Noticeable inhibition in growth of *Klebsiella pneumoniae* after incubation with serum at 2 different dilutions of 10^{-1} and 10^{-3}.

Table 3.4.1: Effect of serum on the inhibition of growth of *Klebsiella pneumoniae*

Date	Serum Initials	Incubation Time (Minute)	CFU without serum	CFU with Serum at Different dilution	CFU with Mg-EGTA	Inhibition % with Serum	Inhibition % with Serum & Mg-EGTA
22.11.21	E	60	10^{-4}=48 colonies CFU=4800000	10^{-2}=124 colonies CFU=2480000	10^{-3}=178 colonies	48.33%	0%
17.01.22	AD	30	10^{-4}=128 colonies CFU=12800000	10^{-3}=52 colonies CFU=10400000		18.75%	
17.01.22	AD	60	10^{-4}=128 colonies CFU=12800000	10^{-3}=37 colonies CFU=7400000	Uncountable	42.96%	0%
17.01.22	AF	30	10^{-4}=128 colonies CFU=12800000	10^{-3}=53 colonies CFU=10600000		17.96%	
17.01.22	AF	60	10^{-4}=128.5 colonies CFU=12800000	10^{-3}=41 colonies CFU=8200000	Uncountable	35.93%	0%
17.01.22	AM	30	10^{-4}=128 colonies CFU=12800000	10^{-3}=48 colonies CFU=9600000		25%	
17.01.22	AM	60	10^{-4}=128 colonies CFU=12800000	10^{-3}=44 colonies CFU=8800000	Uncountable	31.2%	0%

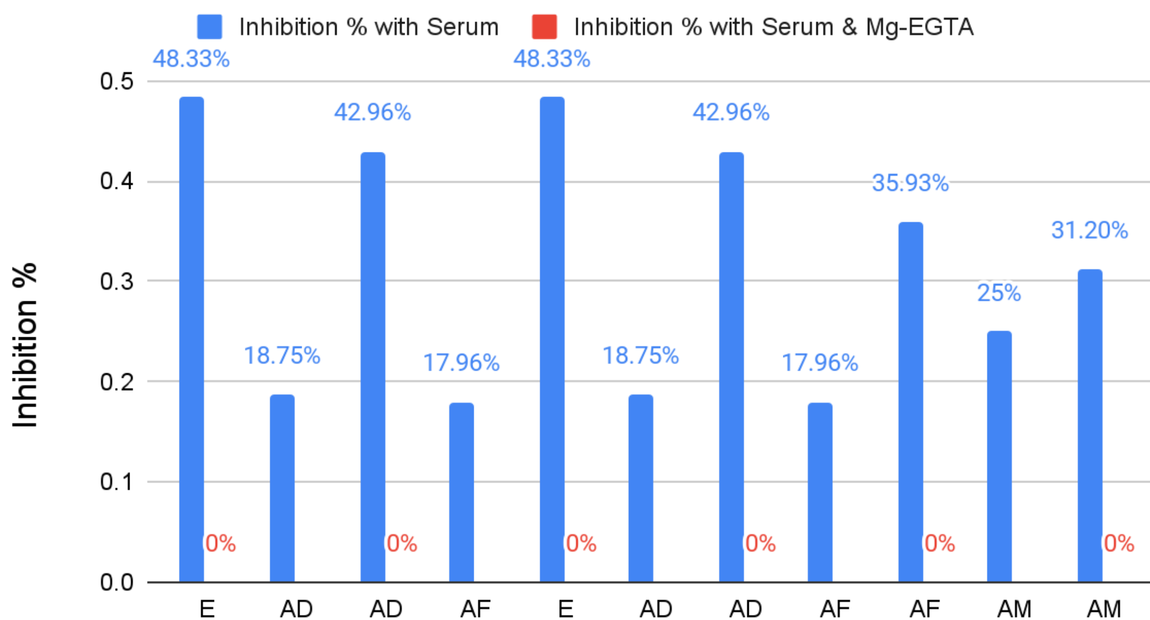

Figure 3.4.2: The inhibition of growth of *K. pneumoniae* with serum and serum + Mg-EGTA respectively.

The inhibition percentage ranged between 17 and 48 percent, as seen in the figure 3.4.2. *Klebsiella pneumoniae*. inhibits at a slower pace than other bacteria. On the other hand, the inhibition percentage is affected by the incubation time for this organism unlike *S. aureus*. We can observe how the inhibition increases by up to 20% when the incubation period is increased by 30 minutes. For all of the individuals, the inhibition rate with serum + Mg-EGTA was 0%, suggesting that it may have blocked the complement pathway necessary to inhibit the growth of bacteria.

3.5 ***Escherichia coli***

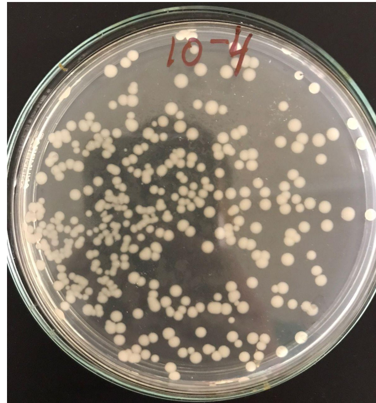

Figure 3.5.1: Growth of *E. coli* after incubation without serum at 10^{-4} dilutions on NA media

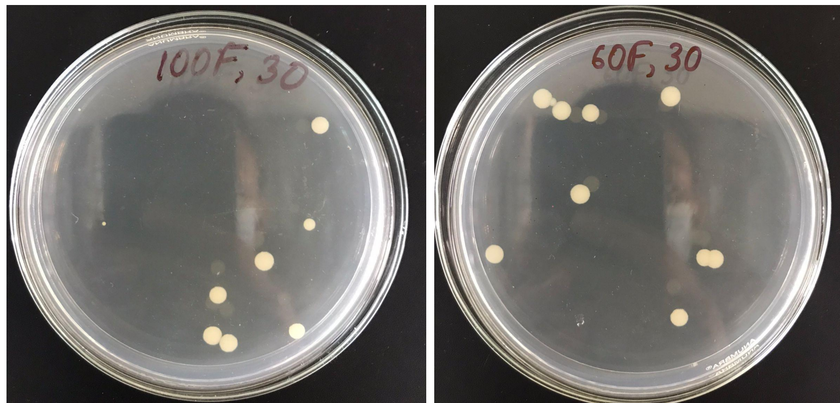

Figure 3.5.2: Noticeable inhibition in growth of *E. coli* after incubation with serum at 2 different dilutions at 10^{-1} and 10^{-3}

Table 3.5.1: Effect of serum on the inhibition of growth of *E. coli*

Date	Serum Initials	Incubation Time (Minute)	CFU without serum	CFU with Serum at Different dilution	CFU with Mg-EGTA	Inhibition % with Serum	Inhibition % with Serum & Mg-EGTA
22.11.21	D	60	10^{-3}=217 colonies CFU=21700000	10^{-3}=37 colonies	10^{-3}=60 colonies	65.89%	44.70%
22.11.21	E	60	10^{-3}=217 colonies CFU=21700000	10^{-3}=51 colonies	10^{-3}=78 colonies	67.82%	50.78 %
30.11.21	B	60	10^{-3}=242 colonies CFU=24200000	10^{-3}=68 colonies	10^{-3}=109 colonies	43.80 %	9.9 %
30.11.21	M	60	10^{-3}=242 colonies CFU=24200000	10^{-3}=76 colonies	10^{-3}=65 colonies	37.19%	46.28 %
4.12.21	D	60	10^{-3}=228 colonies CFU=22800000	10^{-3}=53 colonies	10^{-3}=164 colonies	53.50%	0%
4.12.21	OM	60	10^{-3}=228 colonies CFU=22800000	10^{-3}=70 colonies	10^{-3}=195	38.59%	0%
13.02.22	NA	60	10^{-3}=210 colonies CFU=21000000	10^{-3}=85 colonies	10^{-3}=170	19.04%	0%
13.02.22	AS	60	10^{-3}=210 colonies CFU=21000000	10^{-3}=47 colonies	10^{-3}=175	55.23%	0%
13.02.22	SD	60	10^{-3}=210 colonies CFU=21000000	10^{-3}=50 colonies	10^{-3}=167	52.38%	0%

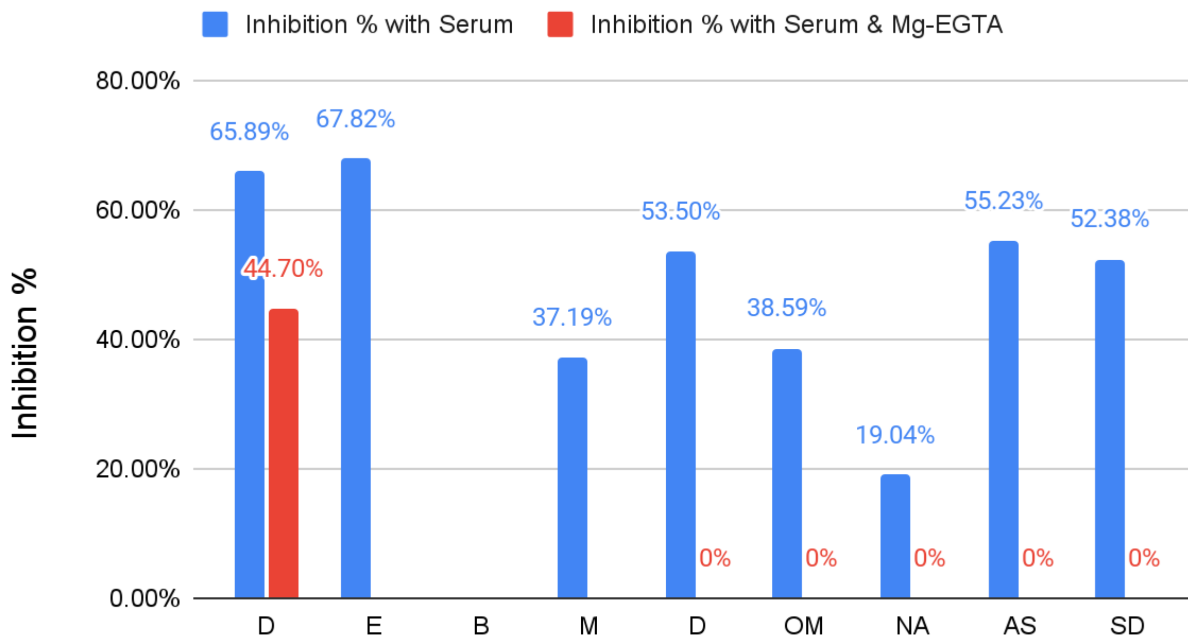

Figure 3.5.3: The inhibition rate of *E. coli* with serum and serum + Mg-EGTA respectively.

The inhibition percentage ranged from lowest 19% to the highest 67%, as seen in the figure 3.3.3. For almost all the individuals, the inhibition rate with serum + Mg-EGTA was 0% except few, suggesting that it may have blocked the complement pathway necessary to inhibit the growth of bacteria.

3.6 *Pseudomonas aeruginosa*

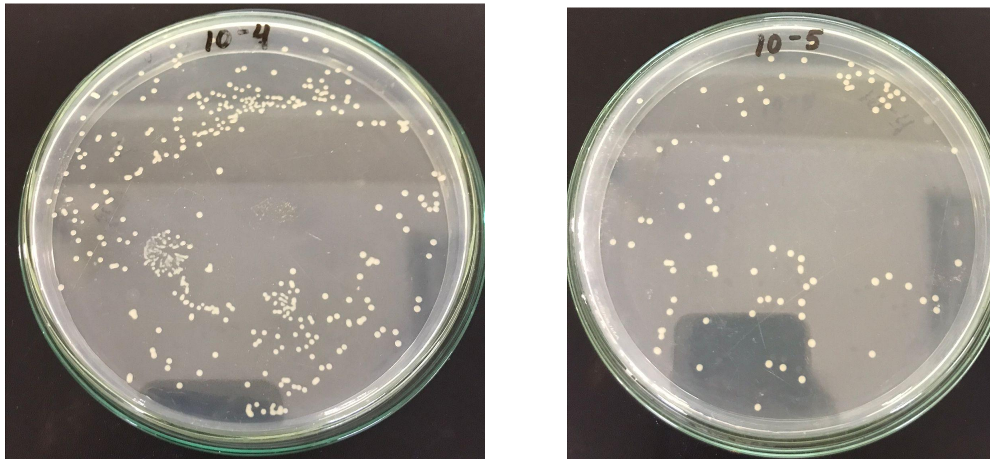

Figure 3.6.1: Growth of *Pseudomonas aeruginosa* after incubation without serum at 10^{-4} and 10^{-5} dilution on Cetrimide media

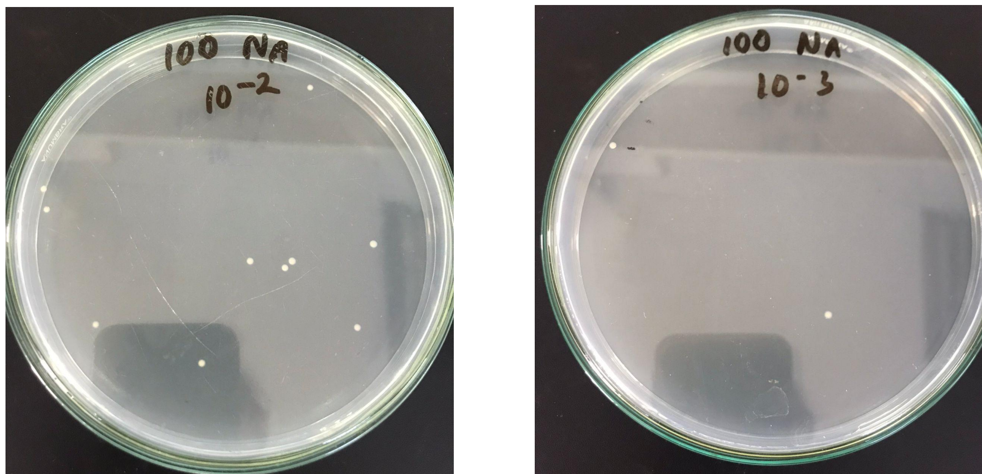

Figure 3.6.2: Noticeable inhibition in growth after *Pseudomonas aeruginosa* incubation with serum at 2 different dilutions at 10^{-1} and 10^{-3}

Table 3.6.1: **Effect of serum on the inhibition of growth of *Pseudomonas aeruginosa***

Date	Serum Initials	Incubation Time (Minutes)	CFU without serum	CFU with Serum at Different dilution	CFU with Mg-EGTA	Inhibition % with Serum	Inhibition % with Serum and Mg-EGTA
26.10.21	MH	60	10^{-4}=120 colonies CFU=12000000	10^{-3}=42 colonies CFU=8400000	10^{-3}=66 colonies	30%	0%
29.11.21	A	60	10^{-4}=116 colonies CFU=11600000	10^{-3}=42 colonies CFU=8400000	Uncountable	27%	0%
29.11.21	A	30	10^{-4}=116 colonies CFU=11600000	10^{-3}=53 colonies CFU=10600000		8.6%	
6.12.21	D	60	10^{-4}=143 colonies CFU=14300000	10^{-3}=35 colonies CFU=7000000	Uncountable	51.04%	0%
6.12.21	OM	60	10^{-4}=143 colonies CFU=14300000	10^{-3}=41 colonies CFU=8200000	Uncountable	42.65%	0%
15.11.21	B	60	10^{-4}=116 colonies CFU=11600000	10^{-3}=33 colonies CFU=6600000	10^{-3}=63 colonies	8.6%	0%
2.12.21	Ifti	60	10^{-5}=140 colonies CFU=140000000	10^{-3}=165 colonies CFU=33000000	10^{-3}=263 colonies	76.36%	0%
2.12.21	SHA	60	10^{-5}=140 colonies CFU=140000000	10^{-3}=222 colonies CFU=44400000	10^{-3}=163 colonies	68.57%	76.71%
27.02.22	TA	60	10^{-5}=78 colonies CFU=78000000	10^{-2} = 51 CFU=1020000		96.55%	
27.02.22	SD	60	10^{-5}=78 colonies CFU=78000000	10^{-2} = 46 CFU=920000		92.24%	
27.02.22	AM	60	10^{-5}=78 colonies CFU=78000000	10^{-2} = 20 CFU=400000		99.56%	
27.02.22	NA	60	10^{-5}=78 colonies CFU=78000000	10^{-2} = 10 CFU=200000		99.13%	

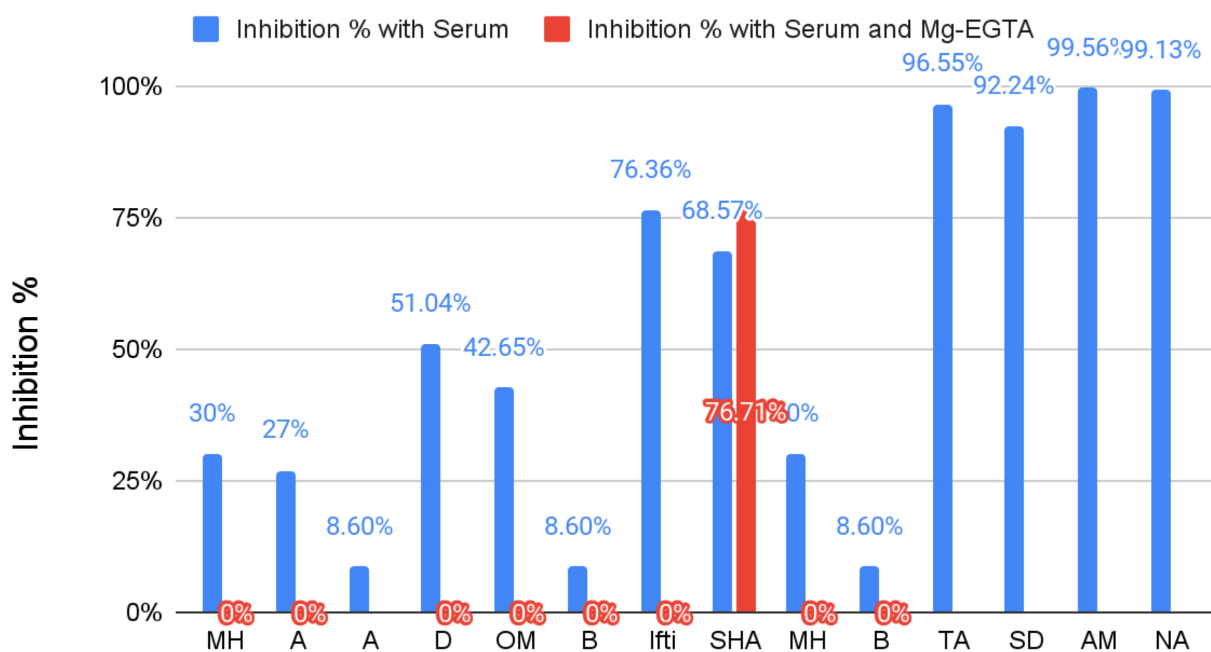

Figure 3.6.3: The inhibition percentage of *P. aeruginosa* with serum and serum + Mg-EGTA respectively.

The inhibition percentage ranged from lowest 8% to the highest 99%, as seen in the figure 3.6.3. The incubation time has a negligible effect on the inhibition percentage.

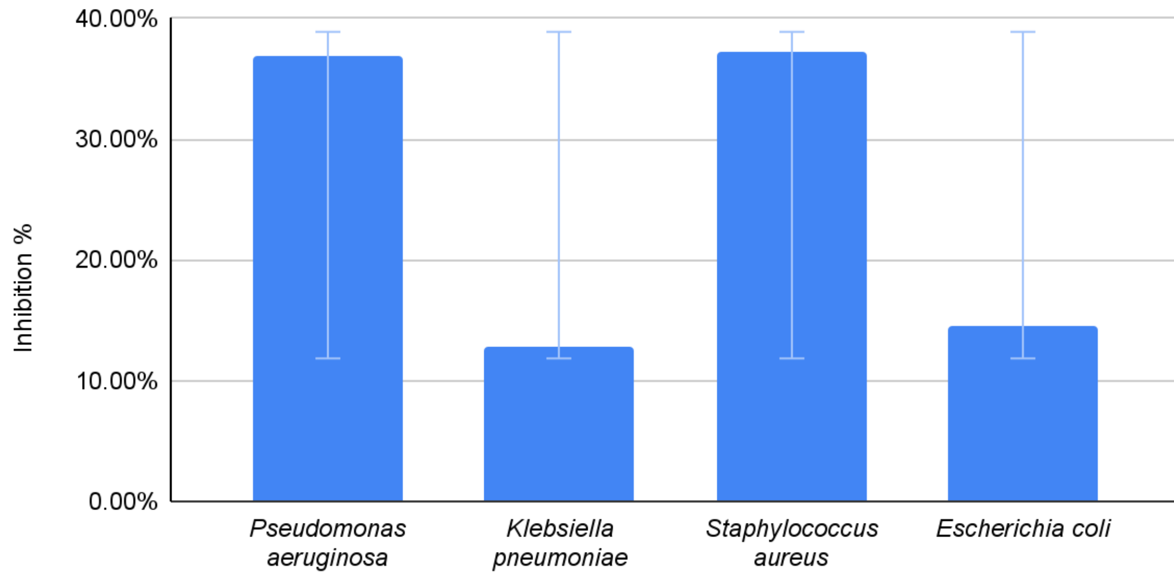

Figure 3.6.4: The inhibition percentage and standard deviation of each organism with serum respectively.

The inhibition percentage of the organisms shows that error bars overlap with each other. This indicates the difference of inhibition among the organism is not statistically significant.

Chapter 4

Discussion

Discussion:

Our initial objective was to see how different incubation times affect the outcome based on prior research where the bactericidal activity of nine *S. typhi* infected human sera was tested against *S. typhi* (Shariar et al, 1970). It was discovered in the study that the activity of not all the organisms showing the same serum mediated killing increased with the increase in incubation time. In some cases, for example, *Klebsiella pneumoniae.* serum mediated killing increased by almost 20% with only 30 minutes of increased incubation. However, for others the effect of increased incubation time was very low and almost negligible. These data suggest that normal human subjects developed significant immunity against MDR bacteria in our country (Langereis et al., 2018).

Previously, many studies were carried out on SBA as the bactericidal effect of normal human serum plays an important role in host defense through their complement mediated pathway against bacterial infection. It helps to gain an understanding of the bacteria and also the host being affected by it and also proved to work as an investigational tool for comparing the respective efficacy of antibiotics administered in combination or alone (Klastersky, 1980). One of the studies on SBA showed that serum mediated killing differs from organism to organism. IgM and IgG classes of immunoglobulins are found to play an important role in complement mediated pathways through cellular lysis of pathogens (Nesargikar et al., 2012).

Experimental models have highlighted the relevance of SBA. Both the bacteria's serum resistance and the host's SBA can impact the outcome of an *E. coli* infection, according to Durack and Beeson. Rabbits with congenital C6 deficiency developed endocarditis following intravenous injections of serum-sensitive or serum-resistant *E. coli* in their studies. Normal rabbits were resistant to serum-sensitive *E. coli* infection, but not to serum-resistant *E. coli* infection (Durack & Beeson, 1977). Similar results were recently found using an *E. coli* meningitis model. These investigations show the role of SBA as a natural defensive mechanism since C6-deficient rabbits lack SBA but have the normal opsonic ability. *E. coli* stays inside the intestinal lumen in normal circumstances and is not harmful. Germs that are normally "noninvasive" may flow out of the intestinal lumen and into the circulation in the presence of portal hypertension; this would be equivalent to the bacteria being directly injected into the experimental animal. If the bacteria are entirely resistant to SBA or if the bacteria are serum

sensitive but the host lacks regular SBA, they are more likely to grow in the circulation which we have found in our *E. coli* results as well.

The requirement for complement pathways in serum mediated killing of MDR organisms was also examined by inactivating both classical pathways of the complement system by adding EGTA. In the majority of instances, this approach resulted in a total loss of serum bactericidal activity (Thomassen and Demko, 1981). In this study we found that EGTA significantly or completely inhibited SBA. This suggests that the classical complement system pathway is important in MDR organism death caused by serum. The complement system appears to be an important aspect of the host's defense against both gram positive and gram-negative bacterial infection, according to a large body of evidence. However, in some cases we also found a lower inhibition percentage with EGTA which also suggests that EGTA was unable to inhibit the SBA. In brief, the current study's findings suggest a significant percentage prevalence, however further research is needed in this area.

Conclusion:

In conclusion, the requirement of the anti-MDR organism antibodies and the consequent activation of classical activation was investigated by examining the bactericidal activity of different human serum. We know, serum bactericidal activity is a useful metric in immunology investigations because it may be used to assess innate defense mechanisms, which can be impacted or controlled by a variety of substances or activities. This study shows that where bacteria are getting multi drug resistant like wildfire in our country, we have to study more about the serum bactericidal activity to strengthen our innate defense.

References:

1. Asna, S. M., Haq, J. A., & Rahman, M. M. (2003). Nalidixic acid-resistant Salmonella enterica serovar Typhi with decreased susceptibility to ciprofloxacin caused treatment failure: a report from Bangladesh. *Japanese journal of infectious diseases*, *56*(1), 32–33.

2. Biswas, M., Roy, D.N., Tajmim, A. *et al.* Prescription antibiotics for outpatients in Bangladesh: a cross-sectional health survey conducted in three cities. *Ann Clin Microbiol Antimicrob* **13,** 15 (2014). https://doi.org/10.1186/1476-0711-13-15
3. Cohen, I. R. and Norins, L. C. 1966. Natural human antibodies to gram-negative bacteria: immunoglobulins G, A, and M. Science. 152, 1257-1259.
4. Dunkelberger, J., Song, WC. Complement and its role in innate and adaptive immune responses. *Cell Res* **20,** 34–50 (2010). https://doi.org/10.1038/cr.2009.139
5. Durack, D. T., & Beeson, P. B. (1977). Protective role of complement in experimental Escherichia coli endocarditis. *Infection and Immunity*, *16*(1), 213–217. https://doi.org/10.1128/iai.16.1.213-217.1977
6. Fierer, J., & Finley, F. (1979). Deficient Serum Bactericidal Activity Against Escherichia Coli in Patients with Cirrhosis of the Liver. *Journal of Clinical Investigation*, *63*(5), 912–921. https://doi.org/10.1172/jci109391

7. Hoque, R., Ahmed, S. M., Naher, N., Islam, M. A., Rousham, E. K., Islam, B. Z., & Hassan, S. (2020). Tackling antimicrobial resistance in Bangladesh: A scoping review of policy and practice in human, animal and environment sectors. *PloS one*, *15*(1), e0227947. https://doi.org/10.1371/journal.pone.0227947
8. Inoue, K., Yonemasu, K., Takamizawa, A., and Amano, T. 1968. Studies on the immune bacteriolysis. XIV. Requirement of all nine components of complement for immune bacteriolysis. Biken. J. 11, 203-206.
9. Isogai, E., Kamewaka, Y., Isogai, H., Kimura, K., Fujii, N., & Nishikawa, T. (1994). Complement-mediated killing of Borrelia garinii--bactericidal activity of wild deer serum. *Microbiology and immunology*, *38*(9), 753–756. https://doi.org/10.1111/j.1348-0421.1994.tb01852.x
10. Klastersky, J. The bactericidal activity in serum and its prognostic clinical value. *Infection* **11,** S93–S96 (1983). https://doi.org/10.1007/BF01645296

11. Klastersky, J., Zinner, S. H.: Synergistic combinations of antibiotics in gram-negative bacillary infections. Rev. Infect. Dis. 3 (1980) 74--83.
12. Langereis, J.D., Henriet, S.S., Kuipers, S. *et al.* IgM Augments Complement Bactericidal Activity with Serum from a Patient with a Novel *CD79a* Mutation. *J Clin Immunol* 38, 185–192 (2018). https://doi.org/10.1007/s10875-017-0474-7
13. Mason, T. G., and Richardson, G. 1981. Escherichia coli and the human gut: some ecological considerations. J. Appl. Bacteriol. 1, 1-16.
14. Michael, J. G., and Rosen, F. S. 1963. Association of "natural" antibodies to gram-negative bacteria with the Xl-macroglobulius. J. Exp. Med. 118, 619-626.
15. Ortiz, V., Isibasi, A., García-Ortigoza, E., and Kumate, J. 1989. Immunoblot detection of class-specific humoral immune response to outer membrane proteins isolated from Salmonella typhi in humans with typhoid fever. J. Clin. Microbiol. 27, 1640-1645.
16. Rajagopalan, P., Kumar, R., and Malaviya, N. 1981. Immunological studies in typhoid fever. 1. Immunoglobulins, C3, antibodies, rheumatoid factor and circulating immune complexes in patients with typhoid fever. Clin. Exp. Immunol. 44, 68-73.
17. Sansano, M. Jr., Reynard, A. M., and Cunningham, R. K. 1985. Inhibition of serum bactericidal reaction by lipopolysaccharide. Infect Immun. 48, 759-762.
18. Shahriar, M., Halder, L. D., Nushrat, N., Hossain, M., Kabir, A. H., & Kabir, S. (2010). Comparison of Bactericidal Activity of Serum Collected from Typhoid Patients and Normal Human Against <i>Salmonella typhi</i> at Various Incubation Time. *Dhaka University Journal of Pharmaceutical Sciences*, *9*(1), 66–67. https://doi.org/10.3329/dujps.v9i1.7436
19. Šterzl, J., Kostka, J., and Lanc, A. 1962. Development of bactericidal properties against gram-negative organisms in the serum of young animals. Folia Microbiol (Prague). 72, 162-174.
20. Taylor, P. W., and Kroll, H. P. 1983. Killing of an encapsulated strain of Escherichia coli by human serum. Infect. Immun. 39, 122-131.
21. Thomassen MJ, Demko CA. Serum bactericidal effect on Pseudomonas aeruginosa isolates from cystic fibrosis patients. Infect Immun. 1981 Aug;33(2):512-8. doi: 10.1128/iai.33.2.512-518.1981. PMID: 6792079; PMCID: PMC350729.

22. Wade, J., Schimpff, S. C.: Antibiotic therapy for febrile granulocytopenic patients. In: Klastersky, J., Staquet, M. J, (eds.): Combination antibiotic therapy in the compromised host. Raven Press, New York 1982, pp. 125-146.

Appendix 1

Media compositions

Mannitol Salt Agar

Component	**Amount (g/L)**
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

MacConkey Agar

Component	**Amount (g/L)**
Peptone (Pancreatic digest of gelatin)	17g/L
Proteose Peptone (meat and casein)	3g/L
Lactose monohydrate	10g/L
Bile Salts	1.5g/L
Sodium Chloride	5g/L
Neutral Red	0.03g/L
Crystal Violet	0.001g/L
Agar	13.5g/L

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Cetrimide Agar

Component	Amount (g/L)
Gelatin Peptone	20.0g/L
Magnesium Chloride	1.4g/L
Potassium Sulfate	10.0g/L
Cetrimide	0.3g/L
Glycerol	10ml/L
Agar	13.6g/L

EMB Agar

Component	Amount (g/L)
Peptic digest of animal tissue	10.000g/L
Dipotassium Phosphate	2.000g/L
Lactose	5.000g/L
Sucrose	5.000g/L
Eosin-Y	0.400g/L
Methylene Blue	0.065g/L
Agar	13.500g/L

Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromothymol blue	0.08

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

Methyl red Voges- Proskauer (MRVP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Mueller-Hinton Agar

Component	Amount (g/L)
Beef, infusion	300.0
Casamino acids	17.5
Starch	1.5
Agar	17.0

Appendix 2

Reagents

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of distilled water was added to make 500 ml of a 0.05% (wt./vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt./vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt./vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature.

Catalase Reagent (20 ml)

3% hydrogen peroxide

Ethyl Alcohol 95%

95 ml of ethyl alcohol (100%) was added to 5 ml of distilled water. This solution was stored at room temperature.

EGTA (Ethylene glycol tetraacetic acid)

For a 100 mM EGTA stock solution, 3.8 g EGTA was added to about 20 ml of distilled H2O and using NaOH it was adjusted to pH11. After that the pH was adjusted to 8.0 with HCl and H2O was added to make a final volume of 100 ml. Filter-sterilize and store at room temperature.

Antibiotics

Name of the antibiotics	Disc content
Meropenem (MRP)	10 mcg
Imipenem (IPM)	10 mcg
Piperacillin (PIT)	30 mcg
Amikacin (AK)	30 mcg
Tetracycline (TE)	30 mcg
Azithromycin (AZM)	15 mcg
Amoxicillin (AML)	10 mcg
Erythromycin (E)	15 mcg
Norfloxacin (NX)	10 mcg
Doxycycline (DO)	30 mcg
Moxifloxacin (MXF)	5 mcg
Cefepime (CPM)	30 mcg
Kanamycin (K)	30 mcg
Nitrofurantoin (NIT)	300 mcg

Appendix 3

Instruments

Instrument/Equipment	**Manufacturer**
Electric Balance	Scout, USA SC4010
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Water Bath	Daihan Scientific Companies, Korea
Table Top Centrifuge	Digi system, Taiwan
Microscope	A. Krüssoptronic, Germany
-20°C Freezer	Siemens, Germany
Vortex Machine	VWR International
Inoculating loop	Tarsons Products, Pvt Ltd, Kolkata
Bunsen burner	Tarsons Products, Pvt Ltd, Kolkata
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Microcentrifuge tubes	Tarsons Products, Pvt Ltd, Kolkata
Conical flask	Tarsons Products, Pvt Ltd, Kolkata
Petri Dish	Tarsons Products, Pvt Ltd, Kolkata
Falcon tubes	Tarsons Products, Pvt Ltd, Kolkata
Vials	Tarsons Products, Pvt Ltd, Kolkata
pH paper	Tarsons Products, Pvt Ltd, Kolkata
Filter and Syringe	Tarsons Products, Pvt Ltd, Kolkata