EFFECTS OF VITAMIN C IN ENHANCING THE ACTIVITIES OF ANTIBIOTICS AGAINST MULTI-DRUG RESISTANT BACTERIA

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

Declaration

It is hereby declared that

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

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Approval

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Abstract

The emergence and proliferation of antibiotic-resistant pathogens have become a global threat. A large number of the pathogens are becoming resistant even to the last resort of antibiotics. The project aimed to study the antimicrobial effect of adjuvants against multi-drug-resistant bacteria by using Vitamin C in three different concentrations. The concentrations are 1.66 mg/ml, 3.33 mg/ml, and 6.66mg/ml. Six isolates of Klebsiella pneumonia were taken. Among them five isolates (KPJANCH1, KPFEBNCH6, KPMARDSH4, H1NONCH, KPFEBDSH2) became susceptible to Amikacin in all three concentrations, among them one isolate H1NONCH became susceptible to Imipenem in all three concentrations, one isolate KPJANCH1 to Amoxicillin + Clavulanic acid (1.66mg/ml, 6.66mg/ml) but KPFEBDSH2 showed susceptibility to Cefixime (1.66 mg/ml and 3.33 mg/ml), two isolates (KPFEBNCH6, KPFEBDSH2) showed susceptibility to Azithromycin, KPFEBDSH2 in all the concentration but KPFEBNCH6 remained resistant in 3.33 mg/ml. KPFEBDSH2 also became susceptible to Ciprofloxacin and Cefixime (1.66mg/ml, 3.33mg/ml). KPFEBNCH6, KPMARDSH4, and KBFEBDSH2 became sensitive to Co-trimoxazole only in all the concentrations and KPMARDSH4 to Ceftriaxone (1.66 mg/ml). Four multi-drug-resistant E. coli isolates were treated with Vitamin C along with antibiotics, one of them remained resistant in all the concentrations by ten antibiotics and the rest of them showed susceptibility in the mentioned concentrations of Vitamin C, two isolates, E213 (6.66 mg/ml) and E238 (3.33 mg/ml) showed susceptibility to Imipenem, E238 also became susceptible to Amoxicillin + Clavulanic acid (6.66 mg/ml). Another isolate 254 by Azithromycin (3.33 mg/ml).

From 6 Multi-drug resistant isolates of Staphylococcus aureus, 4 isolates showed susceptibility. Among them, three isolates (JUDNCH, SA-OTDSH-12, SEPNCW1-1) became susceptible to Amikacin in the first concentration but JUDNCH and SA-OTDSH-12 showed susceptibility at a Vitamin C concentration of 3.33 mg/ml and 6.66 mg/ml as well. For Gentamycin, SA-OTDSH-12 became susceptible in all the concentrations of Vitamin C, and SEPNCW1-1 showed sensitivity at 1.66 mg/ml. Two isolates (SEPDNCW3-15, SEPNCW1-1) became susceptible to Ciprofloxacin in all three concentrations of vitamin C. Lastly, one

isolate (NONCW2-15) became susceptible to Amoxicillin + Clavulanic acid in the last two concentration. SA-SEPCNW-2 showed susceptibility to Ceftriaxone when combined with Vitamin C at a concentration of 6.66mg/ml. However, the result of remnant isolates of Klebsiella pneumonia, E. coli,

and Staphylococcus aureus remained indifferent. Further research is needed to understand the underlying effect of vitamin C as an adjuvant.

Keywords: Antibiotic resistance; Antibiotic Susceptibility testing; Vitamin C; Adjuvant

Dedication

Dedicated to

Our parents, without their love and support we would not be able to finish our undergraduate journey. Each of our beloved family members for their immense support and friends for their consistent motivation.

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Acronyms

Abbreviation	**Elaboration**
AMR	Antimicrobial resistance
AST	Antibiotic Susceptibility Test
MDR	Multi-Drug Resistant
LB	Luria-Bertani
BSI	Blood stream infection
JANIS	Japan Nosocomial Infection Surveillance
UTI	Urinary tract infection
WHO	World Health Organization
AK	Amikacin
AMC	Amoxicillin-clavulanic acid
IPM	Imipenem
COT	Co-trimoxazole
CFM	Cefixime
AZM	Azithromycin
CTR	Ceftriaxone
CN	Gentamicin
E	Erythromycin
CIP	Ciprofloxacin

Chapter 1

Introduction:

1.1: Background of antimicrobial resistance and the need for alternative approaches

Antimicrobial resistance is a state that arrives when microbes alter or change their mechanisms of protecting themselves from the effects of antimicrobials. Antimicrobial resistance (AMR) has become a global health crisis as antimicrobial drugs become less effective or resistant than were once commonly used to treat bacterial, fungal, and parasitic infections. There are four distinguishable categories of antimicrobial resistance mechanisms and they are (i) reducing uptake of a drug; (ii) changing a drug target; (iii) making a drug inoperative; (iv) active drug stream (Reygaert W. C., 2018).

The scenario of bacteria getting resistant to antibiotics is a global health crisis reason being the number of bacteria that become resistant to antibiotics is several times more than the number of new antibiotics and the spreading out of resistant bacteria from closed environments such as hospitals or laboratories into large scale population are increasing. Resistance to pathogens is a consequence of selection pressure arising from an antibiotic or any other compound, for example antiseptic. When the pathogen and an antibiotic or any other composite share a minimum one identical mechanism of resistance then the pathogen becomes resistant. Here two conditions should be met. The first one is there should be extended contact between the bacterial population and the selecting substance which is called a selector. The second one is the concentration of the selector must allow the bacteria to survive. This concentration is generally called sub-inhibitory concentration. Most of the strains of *E. Coli, Klebsiella pneumoniae, and Staphylococcus aureus* are multidrug resistant. *Klebsiella pneumoniae* accounts for pneumococcal disease, such as pneumonia. Pneumonia is a deadly disease for children and it is the leading cause of death in Bangladesh, accounting for 13% of deaths among children under the age of five. (UNICEF Bangladesh, 29 January 2020). According to a distinguished study, if drug-resistant illnesses are not treated properly, they will kill an additional 10 million people per year by 2050. (Ashiru, 2015).

Klebsiella pneumoniae causes pneumonia, bloodstream infection, and urinary tract infection (UTI). Like, *Klebsiella pneumoniae, E. Coli O157:H7* is responsible for UTI along with abdominal and pelvic infection and meningitis. *Staphylococcus aureus* is Gram-positive bacteria, *Staphylococcus aureus* is

especially responsible for skin and soft tissue damage such as abscesses (boils), furuncles, and cellulitis.

1.2: Character and Morphology

Morbidity and mortality are two aftermaths of AMR affecting patients. Resistant bacteria increase the percentage of developing serious health issues and death twice compared to non-resistant forms. (Dadgoster, 2019)

Escherichia coli

Escherichia coli was first recognized as a pathogen in 1885. During this year the pioneering Bavarian pediatrician Theodor Escherich was fighting against newborn infant dysentery at the Otto von Bollinger Institute in Munich. At that time, he first isolated *E. coli* from infant stool (Méric et al., 2016). It is a Gram-negative bacterium. It is generally found in the lower intestine of warm-blooded organisms that are capable of maintaining a certain internal temperature. It is a rod-shaped, non-sporulating, and facultative anaerobe. People are infected with *E. coli* through polluted water or food, particularly insufficiently cooked ground beef. Most strains of *E. coli* are non-toxic, however, a few strains, for example, *E. coli O157:H7* are responsible for severe bloody diarrhea, vomiting abdominal cramps, and hemolytic uremic syndrome (HUS).

Before, 1993 *E. coliO157:H7* wasn't recognized as an impendence and crucial pathogen, but after a severe outbreak that was related to insufficiently cooked ground beef patties sold from a restaurant, it was perceived as a threatening pathogen. Evidences were established and revealed deaths because of bloodstream infection (BSI) in Japan. Through several studies, it was evident that the epidemiological deaths occurred by BSI ascribed to *Escherichia coli* among inhabitants.

Japan Nosocomial Infection Surveillance (JANIS) collected inclusive data of bacterial culturing and susceptibility tests of different antibiotics, by using this data number of BSI caused by *E. coli* between 2011 and 2017 in Japan was evaluated. The death rate was estimated by using BSI mortality acquired from history in Japan. Nine-thousand forty-four cases of BSI death was reported by *E. coli* and expanded to 14,016 in 2017 (Tsuzuki et al., 2020). In Minnesota estimated 160 to 220 cases of *E. coli O157:H7* are indicated each year. After 2-5 days of infection of *Escherichia coli O157:H7,* some

common symptoms are seen. Such as stomach cramps, bloody diarrhea, little fever, and non-bloody diarrhea. Moreover, HUS arises when the *E. coli O157:H7* toxin destroys red blood cells. It can give rise to kidney failure, and neurologic damage, and even 5 – 10% of HUS can cause death.

Klebsiella pneumoniae

Klebsiella pneumoniae is a Gram-negative bacterium. It is encapsulated, nonmotile bacteria, rod-shaped, and facultative anaerobe. In MacConkey media this bacterium is observed as a mucoid lactose fermenter.

These bacteria are generally found in intestines and feces. *K. pneumonia* infections are "nosocomial" infections. It means *K. pneumonia* infects people when they take treatment in a hospital or health care center. Also, people who have poor immune systems and people who are going under treatment are more likely to get a *K. pneumonia* infection. It is an opportunistic bacterium and has high proneness to become antibiotic resistant. They are non-dangerous when they remain in the intestines or stool but if anyhow, they get access to another part of the body, for example in the lung or other respiratory organ they can cause severe infections. Meningitis, perplexity, mild fever, neck rigidity, and eye sensation in bright lights can be caused by *Klebsiella pneumonia* (Li et al., 2022).

Several strains of *Klebsiella* became exceedingly resistant to antibiotics because of producing a specific enzyme. Carbapenems are a class of broad-spectrum antibiotics and they are known as very efficacious against many multidrug-resistant bacteria. Carbapenem is used to treat many Gram-negative bacteria including *Klebsiella*. *Klebsiella pneumoniae* become resistant to carbapenems when they produce the “carbapenemases” enzyme, and these strains are referred to as the ‘*Klebsiella pneumonia* carbapenemes’ organism (KPC). Unfortunately, carbapenem antibiotics are recognized as the last line of defense against Gram-negative bacteria. Diseases caused by *Klebsiella pneumonia* has a high mortality rate, almost 50% of deaths occur despite antimicrobial therapy, and if the patient is alcoholic and affected with bacteremia then the disease rate can be 100% (Qureshi. S., 2022)

Staphylococcus aureus

It is a Gram-positive round-shaped bacterium that has more than 30 types. *Staphylococcus aureus* is a facultative anaerobe bacterium that can grow without oxygen. It was first discovered in 1880 from the

pus of a wound infection. It is normally found in the environment as well as human flora but normally it doesn't cause infection on healthy skin. *Staphylococcus aureus* group of bacteria that can cause various infections such as skin infections, food poisoning, pneumonia, bacteremia, bone infections, toxic shock syndrome, etc. Though this bacterium has different types; *Staphylococcus aureus* is the reason behind most of the infections.

Staphylococcus aureus can be deadly if this pathogen enters the bloodstream, heart, lung, or bone then it can be deadly. It is estimated that in the USA, around 119,000 people have suffered from bloodstream staphylococcus aureus infections in 2017. *Staphylococcus aureus* is a common cause behind both healthcare-associated and community-acquired bloodstream infections. A study conducted in 2019 showed that *staphylococcus aureus* is one of the leading pathogens which is behind 54.9% of global deaths in 2019 (Dall,2022). Furthermore, in 2019, *Staphylococcus aureus* was associated with more than 1 million deaths (Ikuta et al., 2022) A study conducted at Kyoto Medical University found that *S. aureus* has a mortality rate of around 20%–30% and MRSA bacteremia mortality rate is even higher (20%–50%) (Shimizu et al., 2022).

1.3: Antibiotic:

Antimicrobial Resistance:

In the present time, antimicrobial resistance has become a global threat. Antimicrobial resistance (AMR) cancels the effectiveness of antibiotics and increases the risk of death due to infections (Prestinaci et al., 2015). That's why due to drug resistance it is becoming quite impossible to treat. Antimicrobial resistance (AMR) happens when viruses, bacteria, fungi, and parasites evolve and antibiotics cannot work against them. The alarming thing is the overuse as well as misuse of antibiotics are reasons behind developing resistance among bacterial pathogens which causes the cancelation of many traditional treatments. The risk of death due to infections is increasing as well. A study conducted in 2019 estimated that 4.95 million deaths were associated with bacterial AMR.

Table 1: Classes of antibiotics used in this study

Antibiotic Class	**Antibiotic Agent (Himedia®)**	**Symbol**	**Disc Content** (mcg/disc)
Aminoglycosides	Gentamycin	**P**	10
	Amikacin	**AK**	30
Carbapenems	Imipenem	**IPM**	10
Cephalosporin	Cefixime	**CFM**	5
	Ceftriaxone	**CTR**	30
Fluoroquinolones	Ciprofloxacin	**CIP**	5
Macrolides	Azithromycin	**AZM**	30
Macrolides	Erythromycin	**E**	15
Sulfonamides	Co-trimoxazole	**COT**	25
Penicillin + beta lactamase	Amoxicillin and clavulanic acid	**AMC**	30

1.4: Functions of adjuvants in antimicrobial activity

The rate of new antibiotic discovery is very low because of the close-fitting requirement of drug design. The majority of new drug yield procedures halt in the middle and cannot be utilized because of the lack of effective drug design. In this situation, it is very important to alter the implementation of antibiotics according to diverse mechanisms of resistance to existing drugs and target them to enhance antimicrobial efficacy. Multiple target accomplishment has been achieved by combination therapy,

collateral sensitivity, adding adjuvants, hydrolysis of antibiotics, target modification for mutation, and all. A successful approach is adding adjuvants, adjuvants are nonantibiotic compounds that can combine with obsolete antibiotics to target bacterial resistance and enhance their therapeutic state. The concept of "antibiotic adjuvants" has earned significant success in recent times, for example, β-lactamase inhibition mechanisms have been explored.

Antibiotic adjuvants reduce or directly block resistance mechanisms so that the efficiency of existing antibiotics can be facilitated. Antibiotic adjuvant is used experientially for attaining synergistic effect, prevailing resistance, and enhancing spectrum activity. However, adjuvants are nonantibiotic compound, so alone they have no or a bit antimicrobial activity. Antibiotic adjuvants are distinguished into three broad categories depending on their target profile, they are direct, indirect, and host-modulating resistance breakers. They potentiate antibiotics by targeting different active and passive resistance mechanisms in bacteria (Dhanda et al., 2023).

1.5: Rationale for examining vitamin C's effect as adjuvant:

Ascorbic acid or vitamin C is an essential micronutrient that plays an important role in various physiological processes, such as immune function. As it possesses antimicrobial properties it could act as an adjuvant for increasing the efficacy of antibiotics. A handful of studies showed that Vitamin C has direct antimicrobial activity on several pathogens. Moreover, Vitamin C enhances immune cells' activity for killing pathogens which influences the host immune response. By enhancing the immune response, Vitamin C synergizes with antibiotics to combat bacterial infections more effectively. Also, Vitamin C increases the penetration rate of antibiotics into bacterial cells.

To assess the impact of Vitamin C on the zone of inhibition of antibiotics, standardized susceptibility testing methods need to be done. Results acquired from susceptibility tests would reveal the role of Vitamin C in the antimicrobial activity of antibiotics which will establish a foundation for further research. Clinical application knowledge and process mechanisms of ascorbic acid's adjuvant effect against several bacteria including *Staphylococcus aureus, E. Coli,* and *Klebsiella pneumonia* will be facilitated (Chen et al., 2005).

1.6 Objectives:

Evaluating ascorbic acid’s impact as an adjuvant on antimicrobial activity of antibiotics:

- The main objective of this study was to evaluate the potential effect of ascorbic acid on the antimicrobial activity of antibiotics as adjuvant.

- Another objective of this study was to assess the impact of Vitamin C on the susceptibility of specific pathogens, including *Staphylococcus aureus, E. Coli, and Klebsiella pneumonia* to different antibiotics.

- To overcome the Antimicrobial-resistance situation by exploring an alternative approach.

Chapter-2

Materials and Methods

In the present day, many bacteria have changed their survival mechanism and become resistant to antibiotics. Assessment of the antibacterial effects of ascorbic acid is the aim of the current study. By doing Antibiotic Susceptibility Testing of isolates taken from hospitals, communities, and environments highly antibiotic-resistant bacteria are selected.

A total of 16 isolates of *Klebsiella pneumoniae* (6), *E. coli* (4), and *Staphylococcus aureus* (6) were taken for conducting AST to 10 different antibiotics. Highly antibiotic-resistant isolates were exposed to commercially available antibiotics to study the change in antibiotic-resistant patterns. For this, without vitamin C, as well as antibiotics with vitamin C were tested during the research procedure. On nutrient agar, the samples were incubated and grown after being isolated. Then antibiogram was conducted for 10 different antibiotics from 7 different classes.

2.1. Working Laboratory

The present study was conducted in the Biotechnology and Microbiology laboratories of the

Department of Mathematics and Natural Sciences, BRAC University.

2.2 Apparatus and Reagents

Apparatus	**Reagents**
Petri dish	Nutrient agar
Test tube	Agar
Vortex meter	Muller Hinton agar
Eppendorf tube	Ascorbic acid
Bunsen burner	Sodium chloride
Spirit lamp	LB broth

Glass rods	70% ethanol
Micropipette	
Forceps	
Water bath	
Laminar air flow	
Incubator	
Loop	
Needle	
Cotton swab	
Autoclave	
Balance	
Spatula	
Foil paper	
Conical flask	

2.3 Sample Collection

Clinical isolates of Gram-positive and Gram-negative bacteria were collected from hospital drainage and community tap water. Three *Klebsiella pneumonia* isolates and a *Staphylococcus aureus* isolate from Dhaka Shishu Hospital (DSH), two *Klebsiella pneumonia* and a *Staphylococcus aureus* from National Institute of Cancer Research and Hospital (NCH), four *S. aureus* and a *K. pneumonia* were from community tap water (W) and lastly a *S. aureus* was collected from Dedicated Covid-19 Hospital, Mohakhali (DNCH). Four *E. coli* samples were collected from the environment. A total of 16 isolates were collected from hospitals and community tap water. These clinical strains of *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Escherichia coli* were used for the study. The samples were taken to the BRAC University laboratory for conducting experiments.

Hospital Sample

Staphylococcus aureus and *Klebsiella pneumonia* clinical samples have been collected from drainage water of Dhaka Shishu Hospital (DSH), National Institute of Cancer Research and Hospital (NCH) and Dedicated Covid-19 Hospital, Mohakhali (DNCH). All the samples were taken in a sterilized Duran bottle carefully to avoid air contamination. Then it was brought to the BRAC University lab. Then samples were diluted and cultured on an NA plate using the spread plate method. After incubation, growth was observed in the NA plate and then isolates were taken for further procedure. After 24 hours of incubation, the samples were sub-cultured one more time on the NA plate by using the streak plate method and kept for overnight incubation. After getting inceptive growth each sample went through clarity testing using several biochemical procedures. For continuing the culture process samples were streaked on selective media as well, such as *E. coli* and *Klebsiella pneumonia* isolates were streaked in Sorbitol-MacConkey agar plates and *Staphylococcus aureus* isolates were streaked in Mannitol salt agar. For avoiding impurity, isolates were grown in selective media. Furthermore, to keep stock, the samples were kept at -20°C on T1N1 agar with paraffin oil.

Community sample

Four *S. aureus (*SEPDNCW3-15, (SA-SEPCNW2, NONCW2, SEPNCW1-1) and a *K.*

pneumonia (AUNCW2) samples were collected from community tap water. Here, tap water was collected in a sterilized Duran bottle and taken to the BRAC University lab by taking the required precautions. Then filtration of the sample occurred in 0.45 μm pore size filter membrane paper. After that, it was enriched in BPW broth and kept for overnight incubation. The samples were diluted after being taken from incubation and inoculated in the NA plate using the spread plate method. Then isolates were taken for further procedure.

Environment Sample

Four *E. coli* isolates were taken from the environment sample. The existence of *E. coli* in waters has been considered as an indicator of fecal contamination. Several recent studies have shown that some specific *E. coli* strains can survive and potentially proliferate in the extra-intestinal environment for long periods. This indicates that *E. coli* can become incorporated into endemic microbial groups in the

environment. *E. coli* (213, 235, 238, 254) samples were taken from environmental water bodies and isolates were taken by using the same method as the hospital sample.

2.4 Workflow of sample collection

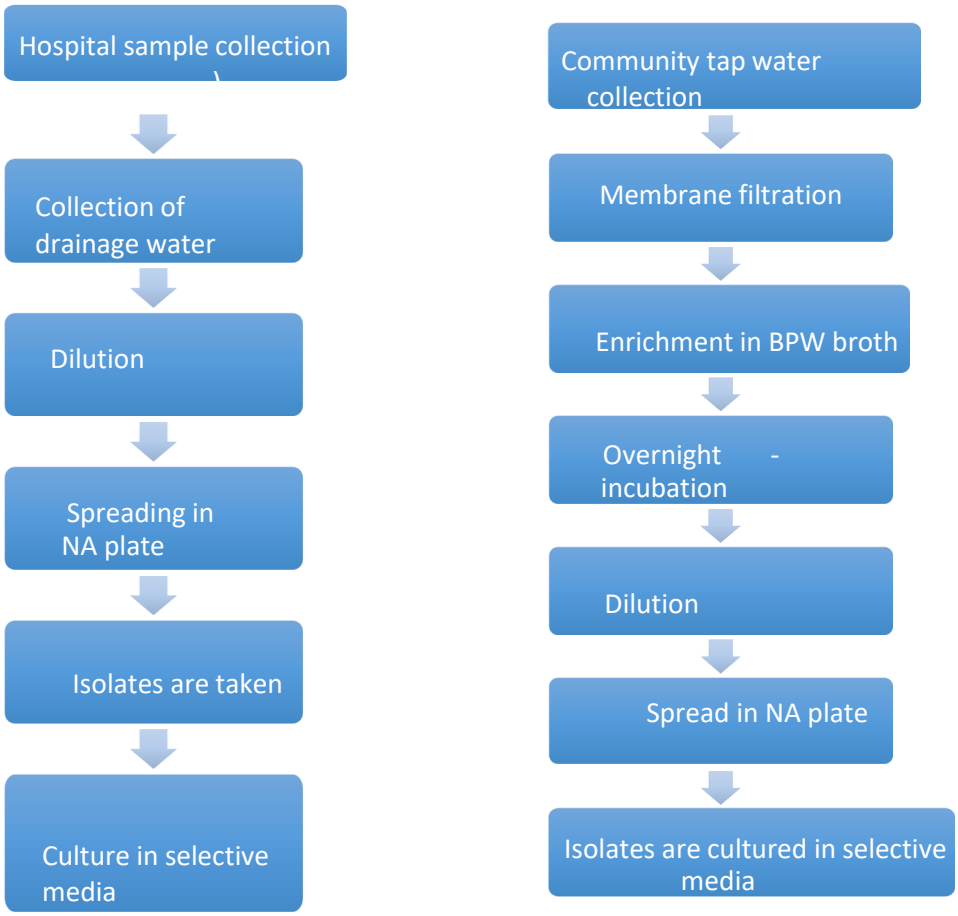


Figure 1: Consecutive Workflow of hospital and community sample collection

2.5 Culture media preparation

Different types of media were used for different purposes in this study.

Table 02: Types of media used

Media	Purpose
T1N1 agar	Used for bacterial stock preparation
Nutrient Agar (NA)	Used for bacterial culture growth and sub-culture
Mueller Hinton agar (MHA)	Used for the determination of susceptibility of bacteria to different antibiotic agents.
Muller Hinton agar + ascorbic acid	Used for determination of the antimicrobial effect of ascorbic acid
Luria Bertani broth (LB)	Used for liquid cultivation and maintenance of bacterial isolates.
Buffered peptone water (BPW)	Used for enrichment purposes of the sample

2.5.1. Composition of T1N1 Agar

At first 10 gm of tryptone, 10 gm of NaCl, and 20 gm of agar powder were measured by using digital balance for making 1000 ml of T1N1 agar. Then required amount of distilled water was added and the mixture was boiled for dissolving appropriately.

2.5.2. Composition of Nutrient Agar

For making 1000 ml of NA 28 g Nutrient agar powder is needed which is a standard measurement. Then the media was boiled and autoclaved at 121° C for 15 minutes for sterilization. After that media is poured into plates for polymerization.

2.5.3. Preparation of LB Broth

Forty grams of LB is the standard measurement for 1000 ml of LB broth. At first LB powder is measured and distilled water is added and boiled for the proper mixture. Then autoclaved at 121° C for 15 minutes and poured into a test tube.

2.5.4. Preparation of Mueller Hinton Agar (MHA) and MHA + ascorbic acid

The methodology of this scientific study conducted an antibiotic susceptibility test on microorganisms using two forms of Muller-Hinton (MHA) media, one with ascorbic acid and the other without. The MHA media without vitamin C acted as the control in the experiment. Three different concentrations of ascorbic acid (1.66 mg/ml, 3.33 mg/ml, 6.66 mg/ml) were made. The media was diluted in the following way:

The concentration of vitamin c was 100 mg/ml

In 147.5 ml of MHA media, 2.5 ml of vitamin c was added.

The final concentration of MHA + vitamin c = 250 mg/150 ml

= 1.66 mg/ml

Similarly, in 145 ml of MHA media, 5 ml of vitamin c was added.

The final concentration of MHA + vitamin c = 500 mg/ 150 ml

= 3.33 mg/ml

Also, in 140 ml of MHA media, 10 ml of vitamin c was added.

The final concentration of MHA + vitamin c = 1000mg/150 ml

= 6.66 mg/ml

Initially, two conical flasks were prepared, each containing the same amount of MHA powder, which was subsequently dissolved in distilled water. The standard form of the usual MHA agar is 38.0 g for 1000 ml of distilled water. Then media was boiled and autoclaved at 121° C for 15 minutes.

After the autoclaving process, one of the media samples was cooled down to 40 °C because vitamin C is heat sensitive, and loses its efficacy at higher temperatures.

1.66 mg/ml of vitamin C: Then 2.5 ml of vitamin C of concentration 100 mg/ml was added by using a micropipette in 147.5 ml of MHA and was mixed properly.

3.33 mg/ml of vitamin C: To prepare the concentration of 3.33 mg/mL 5 mL of Vitamin C of concentration 100 mg/mL was added to 145 mL of MHA and mixed properly

6.66 mg/ml of vitamin C: To prepare Vitamin C concentration of 6.66 mg/mL 10 mL of Vitamin C of concentration 100 mg/mL was added to 140 mL of MHA

It was crucial to carefully maintain the temperature to prevent the media from becoming either too cool, which will cause the media to become solid, or too hot, which would obstruct the functionality of ascorbic acid. The cooled media sample was then prepared for antimicrobial susceptibility testing (AST). Both the MHA media without vitamin C and the cooled MHA media with vitamin C were poured into separate plates. When the media becomes solidified, it provides a suitable substrate for the AST. By using these two forms of MHA media, the antimicrobial susceptibility of the microorganisms under the influence of ascorbic acid can be compared. The use of a control group allowed for a comparative analysis while maintaining the appropriate temperature during cooling and careful polymerization ensured accurate and reliable results for the subsequent antimicrobial susceptibility testing.

2.6: Bacterial stock preparation

Bacterial stock was extremely important for long-term preservation and further use. For bacterial stock preparation, T1N1 agar was prepared and bacteria were inoculated by stabbing 3 times. After that, the vials were incubated at 37 °C for 24 hours. After incubation, 150 microliter paraffin oil was added to the surface of the agar media.

Besides, in another way, the bacterial stock was preserved. In this method, LB medium was used and this method was applied for short-time preservation. For this, in 1 ml LB medium, the bacterial colony was suspended by using a sterile medium and incubated at 37 °C for 24 hours. Later, around 150 microliter of 40% glycerol was added and stored at -20 degrees Celsius.

2.7 : Sub-culture:

For Antibiotic susceptibility testing, a 24-hour fresh culture was necessary. In the sub-culture technique, the bacterial colony is transferred into a new fresh growth medium. With the help of a sterile loop, a single colony was taken and then streaked on a fresh growth medium (NA). After that, the plates were incubated for 24 hours at 37 degrees Celsius.

2.8 : Antibiotic Susceptibility test

Our main aim was to detect multi-drug resistant isolates from the samples collected from different hospitals. We have used a total of 10 antibiotics for both Gram-positive and negative pathogens. Throughout the process, we have followed CLSI guidelines 2019. Detailed information regarding the 10 antibiotics is given below:

Table 3: Ranges of antibiotics

Interpretative criteria (mm)									
	Staphylococcus aureus			***E. coli***			***Klebsiella pneumonia***		
Antibiotics	***S***	***I***	***R***	***S***	***I***	***R***	***S***	***I***	***R***
Gentamycin	**≥ 15**	*13-14*	**≤ 12**	**≥ 26**	*20-25*	**≤ 19**	**≥ 15**	**13-14**	**≤ 12**
Amikacin	**≥ 18**	-	**≤ 18**	**≥ 25**	*19-24*	**≤ 18**	**≥ 18**	**15-17**	**≤ 15**
Imipenem	**≥**	-	**≤**	**≥ 26**	-	**≤ 25**	**≥ 23**	**20-22**	**≤ 19**

Cefixime	≥	-	≤	≥ 27	*20-26*	≤ 19	≥ 19	16-18	≤ 15
Ceftriaxone	≥ 21	*14-20*	≤ 13	≥ 36	*29-35*	≤ 28	≥ 23	20-22	≤ 19
Ciprofloxacin	≥ 21	*16-20*	≤ 15	≥ 30	-	≤ 29	≥ 26	22-25	≤ 21
Azithromycin	≥ 18	*14-17*	≤ 13	≥ 13	-	≤ 12	≥ 13	-	≤ 13
Erythromycin	≥ 23	*14-22*	≤ 13	≥	-	≤	≥ 15	-	≤
Co-trimoxazole	≥ 17	*14-16*	≤ 14	≥ 29	-	≤ 23	≥ 16	11-15	≤ 10
Amoxicillin and clavulanic acid	≥ 26	19-25	≤ 18	≥ 24	-	≤ 18	≥ 19	-	≤ 19

2.9 : Culture preparation: For conducting an antibiotic susceptibility test 24-hour fresh culture was needed. So, we used the 24-hour fresh culture of the pathogens. From the stock, isolates of the 3 pathogens were grown in nutrient agar and incubated for 24 hours at 37 degrees Celsius. Then, a single colony was taken using a sterile loop, added to saline, and vortexed. The turbidity was equivalent to a 0.5 McFarland standard.

2.10 : Inoculation of MHA Plates:

Two types of MHA plates were used. One type was plates containing only MHA and another one was plates containing MHA and ascorbic acid. Also, plates that contain ascorbic and MHA, have 3 different concentrations. To inoculate both types of plates at first a sterile cotton swab was dipped into the bacterial suspension and the swab against the wall of the tube to remove the excess liquid. Then, streaked the plate back and forth motion. To get an even distribution of inoculum, rotate the plate for around 60 degrees each

Placement of the antibiotic disks: Flame-sterilized forceps were used to remove the antibiotic disks from the cartridge. Then, place the antibiotic on the surface of the agar and gently press the antibiotic against the surface. Not more than one antibiotic should be placed at a time. To avoid irregular zone shape, it should be ensured that antibiotic disks are properly attached to the agar surface.

Incubation: After placing the antibiotic disks, place the plates in the incubator and incubate for 18- 24 h at 37 degrees Celsius. Also, plates should be incubated within 15 minutes of placing antibiotic disks.

2.11 : Measuring zone size: With a ruler, the clear circular zone was measured edge to edge over the center of the disk. The diameter of the zone is measured in millimeters. The zones are supposed to be circular and clear. If any uneven zone appears then there must be some error occurred during the experiment.

2.12 : Result and interpretation:

After the conduction of “Antibiotic susceptibility testing, three categories of results can be seen.

- Sensitive: It is a specific zone size range that indicates that the disease caused by that particular pathogen can be treated.
- Intermediate: It is a zone size range that indicates that the disease can be treated but with moderate dosages.
- Resistant: In this condition, no clear zone can be visible. Also, bacteria can grow even if the antibiotic is present.

From the samples, we have used those isolates which are multidrug resistant.

CHAPTER- 3

Results

In this study, Antibiotic Susceptibility Testing was performed to identify the antibiotic-resistant pattern of the isolates and the effect of ascorbic acid in increasing the efficacy of antibiotics. Three antibiotic trials (individual and combination) were tested successfully. The changes in the zone of inhibition after using ascorbic acid as an adjuvant were compared with the Initial AST result to observe any changes in the resistant pattern.

3.1. Initial Antibiotic Susceptibility Testing

Antibiogram Result: After conducting an Antibiotic susceptibility test, a zone of inhibitions was observed both with and without the addition of ascorbic acid in the media. Three different concentrations of ascorbic acid used for this test were 1.66 mg/ml, 3.33 mg/ml, and 6.66 mg/ml. For 16 isolates of three multidrug-resistant pathogens (1 Gram-positive, 2 Gram-negative) 10 antibiotics were used. Results were taken between 18 to 24 hours of incubation.

For the primary screening, Antibiotic Susceptibility Testing of a total of 16 isolates was performed. To elaborate, the resistant pattern of 6 *Klebsiella Pneumonia*, 6 *Staphylococcus aureus*, and 4 *E. coli* isolates was examined for 10 antibiotics.

Table:4-AST for *Klebsiella pneumonia* in the absence of Vitamin C

Sample	Zone of Inhibition (mm) b									
	CN	AK	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
KPJANDSH1	18	0	24	16	21.6	10	19.3	15	18	17
KPFEBNCH6	21.6	0	21	14	16	0	4	23	9	11
AUNCW2	24	20	23.5	25	31	14	20	0	34	23
H1(NONCH)	20	17	21.5	16	29	25	15	10	29	9.5
KPMARDSH4	27	0	35	14	19	10	18	18	9	22
KPFBDSH2	25	0	34.5	15	25	0	10	17	0	27.5

[Red= Resistant, White= Intermediate, Green=Susceptible]

Table 4 shows the susceptibility of *Klebsiella pneumonia* to ten different antibiotics. Among the *K. pneumonia* isolates. 66.7% were resistant, 16.7% were intermediate, and 16.67% were susceptible to AK. 75% were susceptible and 25% were intermediate to IPM. 50% are resistant, 33.33% are intermediate, 16.67% were susceptible to CFM. 66.67% were susceptible, 16.67% were intermediate, and 16.7% were resistant to CTR. 83.33% are resistant, 16.7% were intermediate to CIP. 66.7% were susceptible, 33.33% were resistant to AZM. 66.67% were susceptible, 33.33% were resistant to E. 50% are susceptible, and 50% are resistant to COT. 50% are resistant and 50% are susceptible to AMC.

Table 05: AST for *E. coli* in the absence of Vitamin C

Sample	CN	Ak	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
213	16	19	10	R	R	R	R	R	R	R
235	R	20	15	R	R	R	R	R	R	R
238	R	25	21	R	R	8	18	10	29	R
254	12	24	20.5	R	R	10	12	R	R	R

[Red= Resistant, White= Intermediate, Green=Susceptible]

Table 5 shows the susceptibility of *E. coli* to 10 different antibiotics. 100% were resistant to CN, IPM, CFM, CTR, E, and AMC. 75% of isolates were intermediate to AK 25% were susceptible. 75% were resistant to AZM 25% were susceptible, 75% were resistant to COT and 25% were susceptible.

Table 06: AST for *Staphylococcus Aureus* in the absence of Vitamin C:

Sample	CN	AK	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
SEPDNCW3-15	0	17	16	0	32.5	0	0	0	34	0

JUDNCH	17	15	0	0	18	32	18	0	18	17
SA-SEPCNW2	20.5	25	20	0	0	0	0	0	0	0
SA-OTDSH-12	0	0	14	28	33	21	0	6	30.5	0
NONCW2	15	23	32	0	18	20	16.5	0	25	15
SEPNCW1-1	0	12	15	18	30	R	20	0	30	0

[Red= Resistant, White= Intermediate, Green=Susceptible

The following result shows the susceptibility of *Staphylococcus aureus* to 10 different antibiotics. 50% were resistant and 50% were susceptible in CN. 66.7% were resistant, 33.33% were susceptible in AK. 66.7% were intermediate, 16.7% were R, 16.7% were S. 66.7% were R, 33.33% were I to CFM. 50% were R, 33.33% S, 16.7% I to CTR. 83.3% R, 16.7% to CIP. 50% R, 50% were S to AZM. 66.7% were R, 33.3% I to E. 33.3% were R, 50% S, 16.7% I to COT. 66.7% R, 33.3% I to AMC

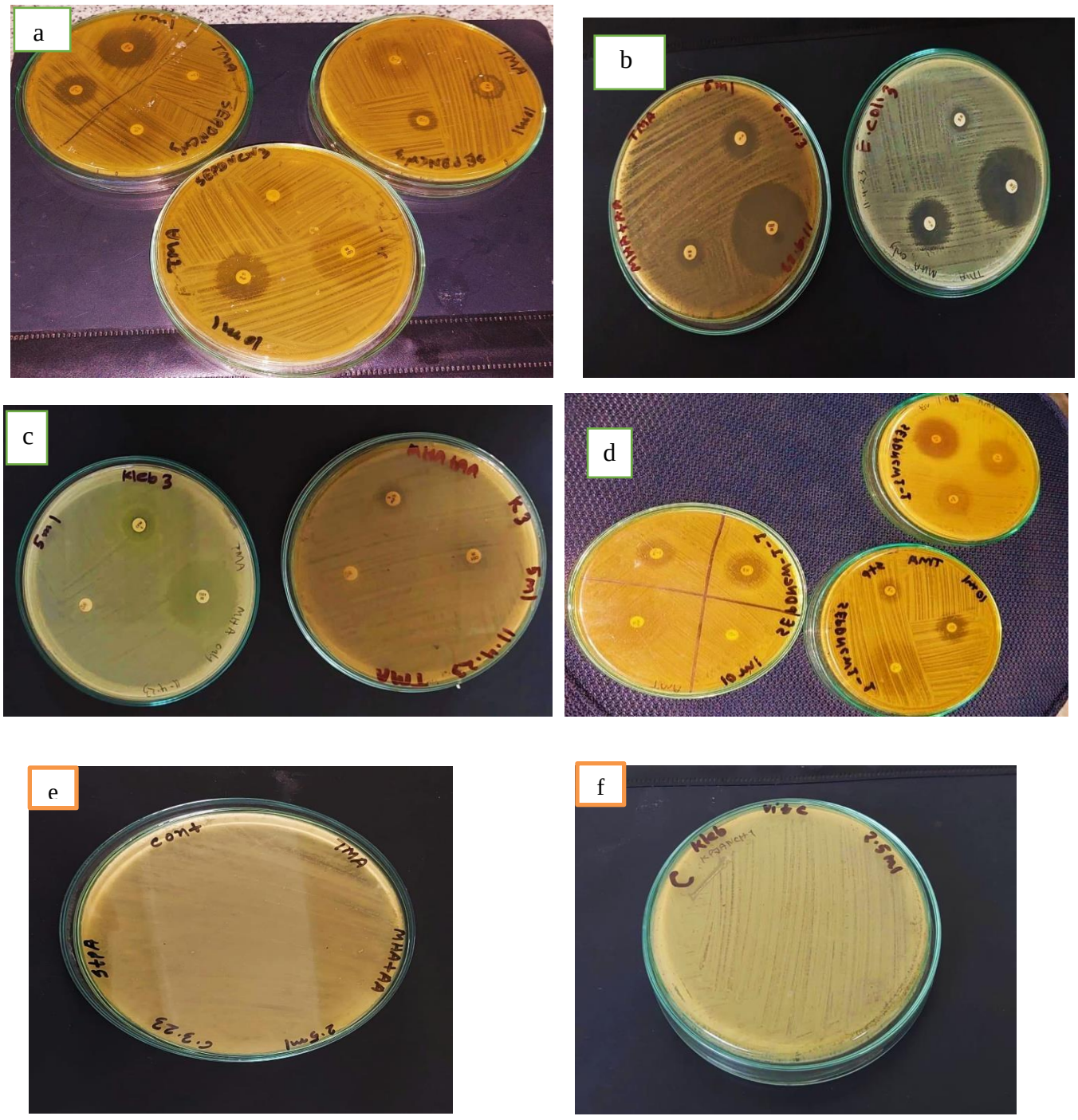


Figure 2: Antibiotic susceptibility testing (Kirby Bauer Disk Diffusion Method) of isolates of three

pathogens in different concentrations of vitamin C. The clear zone size indicate that antibiotics have successfully inhibited bacteria growth; a) SEPDNCW3-15 in 6.66 mg/ml of vitamin C, b) 213 (E. coli) in 3.33 mg/ml, c) KPJANCH1 in 3.33 mg/ml, d) SEPDNCW1-1 in 6.66 mg/ml of vitamin C;

e) Control of *S. aureus* in MHA media with adjuvant; f) Control of *K. pneumonia* in MHA media with adjuvant

This figures (e and f) acted as negative control which proves vitamin C alone does not inhibit the growth fully rather when it was used as adjuvant with antibiotics it enhances the efficacy.

3.2. Adjuvant trials

Here, the bacterial samples were exposed to three different concentrations of ascorbic acid. All the

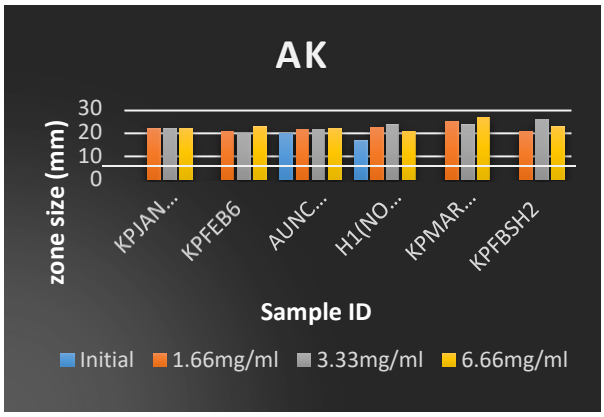
16 bacterial isolates (6 *Klebsiella pneumonia*, 6 *Staphylococcus*, and 4 *E. coli*) were selected for trials. Any changes in the resistant pattern were observed after 24-hour incubation at three different concentrations. This was achieved by the comparison of the initial AST result with 1.66 mg/ml, 3.33 mg/ml, and 6.66 mg/ml these three concentrations of AST result.

Table 7: Comparative analysis of antibiotic resistance pattern of *Klebsiella pneumonia* strains in three concentrations of Vitamin C

Sample ID	CN	AK	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
AST (Initial)										
Zone of inhibition (mm)										
KPJANDSH1	18	0	24	16	21.6	10	19.3	15.3	18	17
KPFEB6	21.5	0	21	14	16	0	4	23	9	11
AUNCW2	24	20	23.5	25	31	14	20	0	34	23
H1(NONCH)	20	17	21.5	16	29	25	15	10	29	9.5
KPMARDSH4	27	0	35	14	19	10	18	18	9	22
KPFDBSH2	25	0	34.5	15	25	0	10	17.3	0	27.5
Antibiotics + vitamin C at 1.66 mg/ml (final concentration)										
Sample ID	CN	AK	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
KPJANDSH1	0	22	32	0	0	23	11	0	36	24
KPFEB6	0	21	19	0	0	10	15	8	34.5	10
AUNCW2	15.5	19	24.5	22	27	23.5	21.75	8	27.5	21
H1(NONCH)	17	22.5	23.5	19.5	26	19	16	0	31	11
KPMARDSH4	20	25	36	0	36	20.5	11	10	30	23.5
KPFDBSH2	20	21	35	35	42	33	19	8	41	30
Antibiotics + vitamin C at 3.33 mg/ml (final concentration)										
Sample ID	CN	AK	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
KPJANDSH1	0	22	29	0	0	20	11	0	35	12

KPFEB6	0	20	20	0	0	8	11	8	33	10
AUNCW2	17	21.5	29	31	25	25	14	0	33	22.5
H1(NONCH)	16.5	21.5	24	10.5	32	17	10	0	29.5	13.5
KPMARDSH4	18.5	24	35	0	0	16	13	7	28.5	20
KPFDBSH2	22	26	38	34	42	33.5	21.5	9	44.5	28.5
Antibiotics + vitamin c at 6.66 mg/ml (final concentration)										
Sample ID	CN	AK	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
KPJANDSH1	0	22	35	0	0	21	11	0	34	25.5
KPFEB6	0	23	13.5	8	0	10	17	8	38	10
AUNCW2	16	22	31.5	24.5	31.5	23	9	7	31	22
H1(NONCH)	17	21	25.5	14.5	31.5	9	12.5	0	29.5	17
KPMARDSH4	24	27	40	0	0	17	18.5	8	32	22
KPFDBSH2	18	23	15	0	20	21	14	11	42	35.5

The comparison of zone sizes among the isolates of *Klebsiella pneumoniae* in 3 three different concentrations of Vitamin C is shown in Table 7 (Green color indicates susceptibility)


AK
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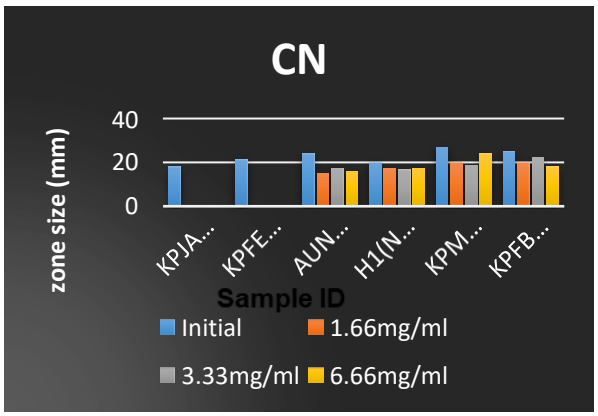
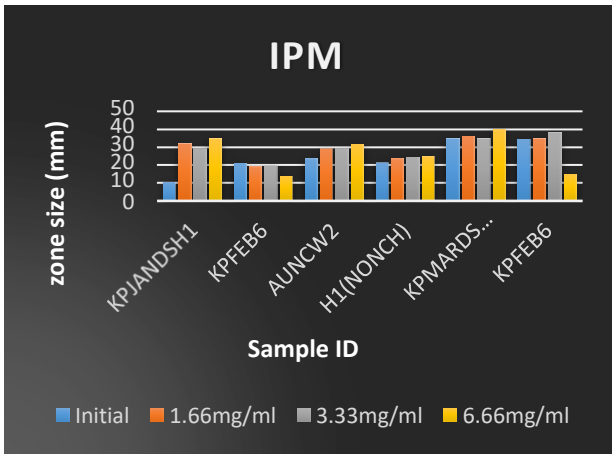
30
20
10
0
KPJAN...
KPFEB6
AUNC...
H1(NO...
KPMAR...
KPFBSH2
Sample ID
Initial
1.66mg/ml
3.33mg/ml
6.66mg/ml

3 (a)


CN
zone size (mm)
40
20
0
KPJA...
KPFE...
AUN...
H1(N...
KPM...
KPFB...
Sample ID
Initial
1.66mg/ml
3.33mg/ml
6.66mg/ml

3(b)


IPM
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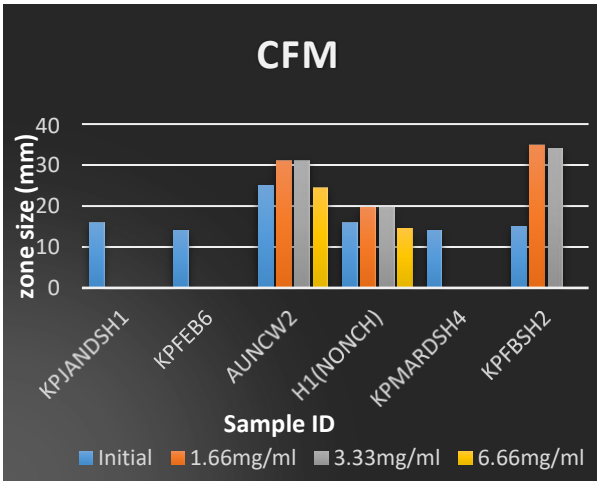
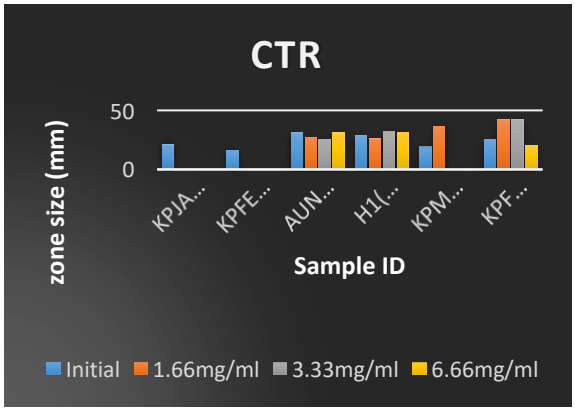
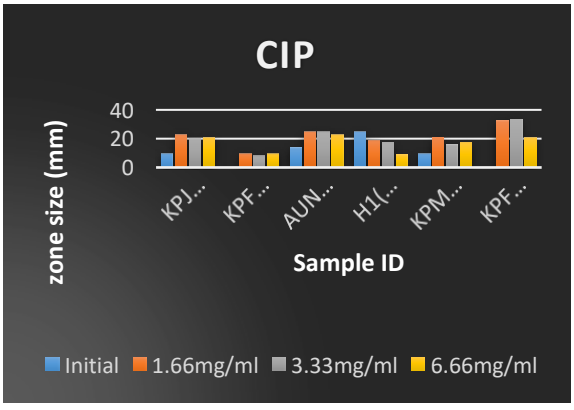
50
40
30
20
10
0
KPJANDSH1
KPFEB6
AUNCW2
H1(NONCH)
KPMARDS...
KPFEB6
Sample ID
Initial
1.66mg/ml
3.33mg/ml
6.66mg/ml

3(c)

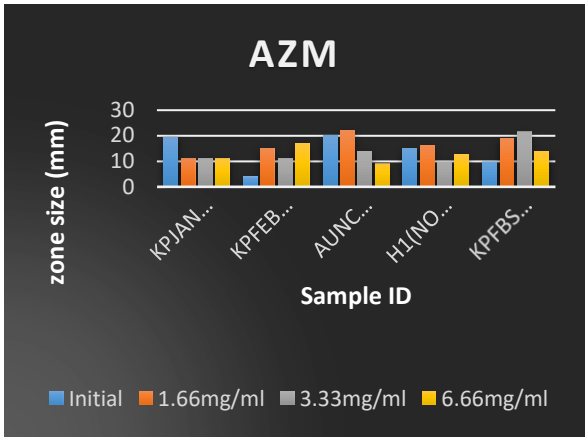

CFM
zone size (mm)
40
30
20
10
0
KPJANDSH1
KPFEB6
AUNCW2
H1(NONCH)
KPMARDSH4
KPFBSH2
Sample ID
Initial
1.66mg/ml
3.33mg/ml
6.66mg/ml

3(d)

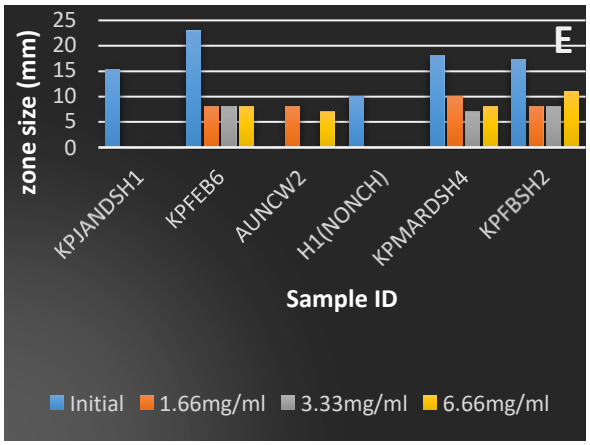

CTR
zone size (mm)
50
0
KPJA...
KPFE...
AUN...
H1(...
KPM...
KPF...
Sample ID
Initial
1.66mg/ml
3.33mg/ml
6.66mg/ml


CIP
zone size (mm)
40
20
0
KPJ...
KPF...
AUN...
H1(...
KPM...
KPF...
Sample ID
Initial
1.66mg/ml
3.33mg/ml
6.66mg/ml

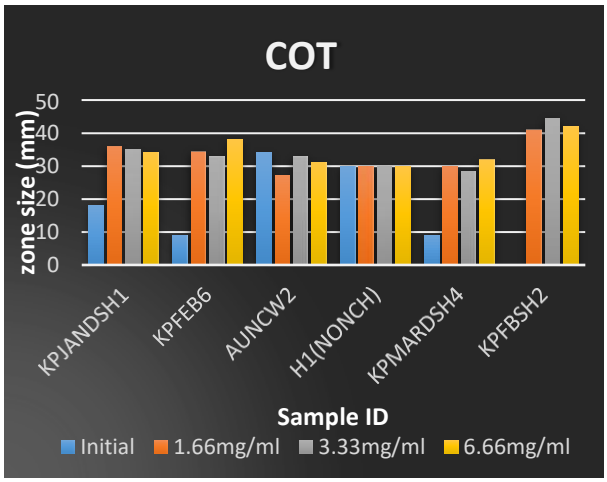
3(e)



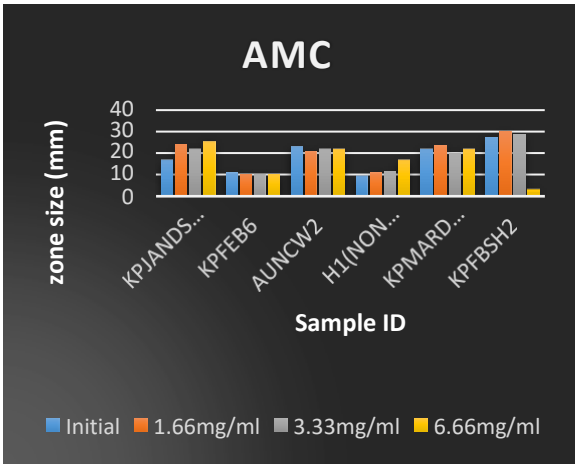
3(f)



3(g)



3(h)



3(i)

3(j)

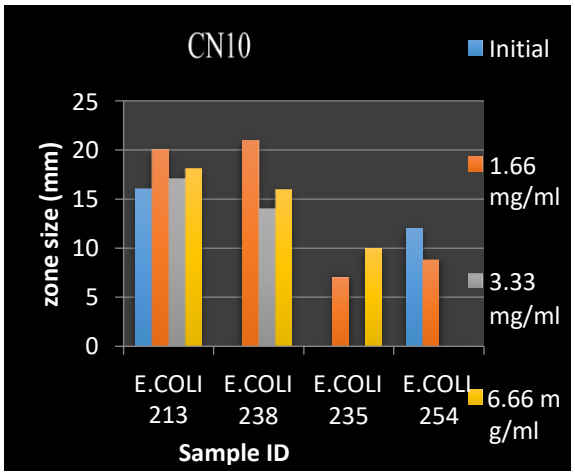
Figure 3: The figure shows a comparative analysis of changes in the zone size of *Klebsiella pneumonia* isolates to antibiotics in three different concentrations (1.66 mg/ml, 3.33 mg/ml, 6.66 mg/ml, mg/ml) of Vitamin C

Table 8: Comparative analysis of antibiotic resistance pattern of *Escherichia coli* strains in three concentrations

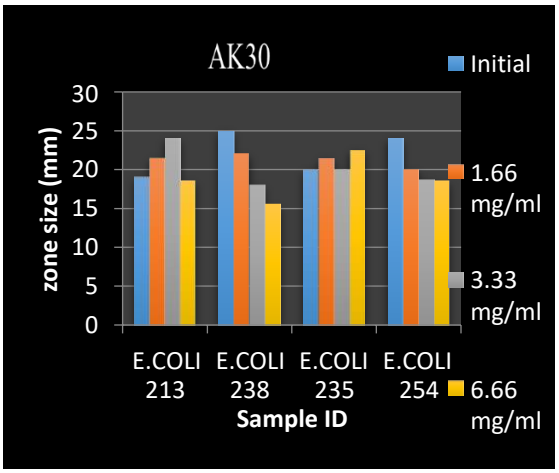
Sample ID	CN	Ak	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
			AST (Initial)							
			Zone of Inhibition (mm)							
213	16	19	10	0	0	0	0	0	0	0
235	0	20	15	0	0	0	0	0	0	0
238	0	25	21	0	0	8	18	10	29	0
254	12	24	20.5	0	0	10	12	0	0	0
			Antibiotics + vitamin c at 1.66 mg/ml (final concentration)							
			Zone of Inhibition (mm)							
213	20	21.5	23	0	0	0	0	0	0	8
235	7	21.5	12	0	8	0	7	0	8	0
238	21	22.5	24	22	12.5	13	7.5	7.5	7	7
254	8.75	20.5	19.25	16.75	24.75	0	14.25	0	0	11.25
			Antibiotics + vitamin C at 3.33 mg/ml (final concentration)							
			Zone of Inhibition (mm)							
213	17	24	25	0	0	0	0	0	0	11.5
235	0	20	16	0	0	0	0	0	0	0
238	14	18	30.5	20	9	13	11	R	26.5	16
254	0	18.75	20.5	14.5	27.5	7	13	0	0	10
			Antibiotics + vitamin C at 6.66 mg/ml (final concentration)							
			Zone of Inhibition (mm)							
213	18.5	18.5	28.75	0	0	0	0	0	0	11.5

235	10	22.5	15	0	0	0	0	0	0	0
238	16	15.5	21	20	29	0	20.5	0	31.5	36.5
254	0	18.5	20.5	18.75	33	0	0	0	0	20

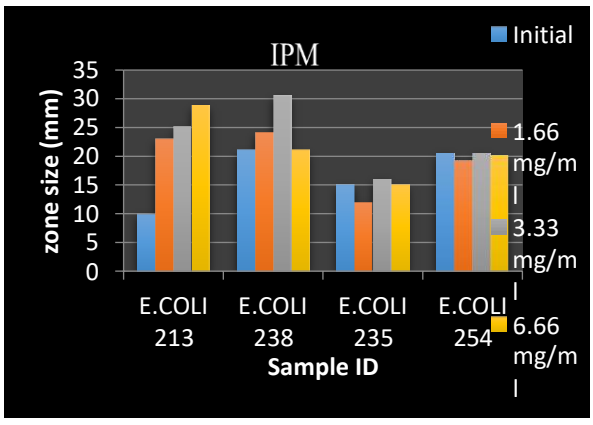
The comparison of zone sizes among the isolates of *E. coli* in 3 three different concentrations of Vitamin C is shown in Table 7 (Green color indicates susceptibility)



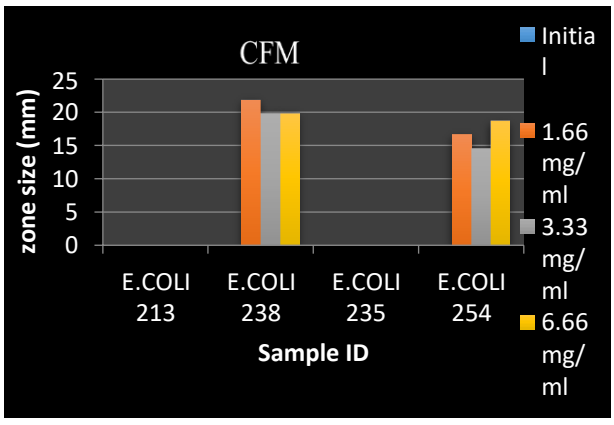
4(a)



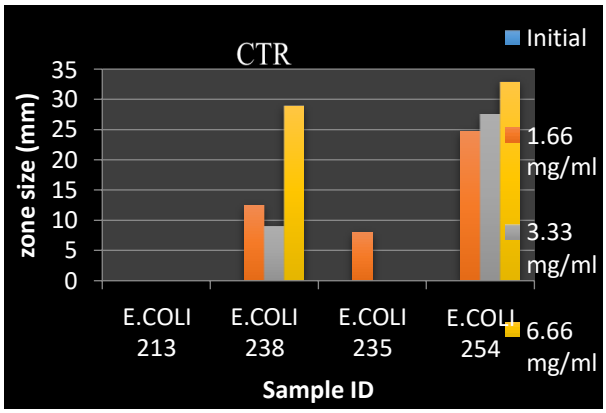
4(b)



4(c)



4(d)



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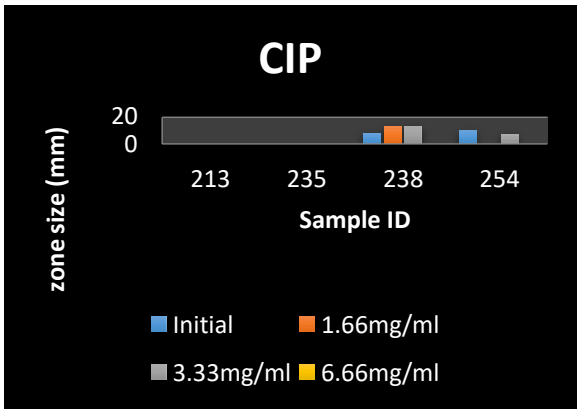

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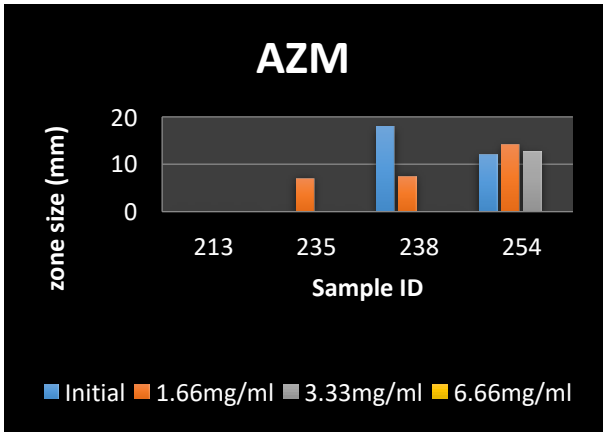

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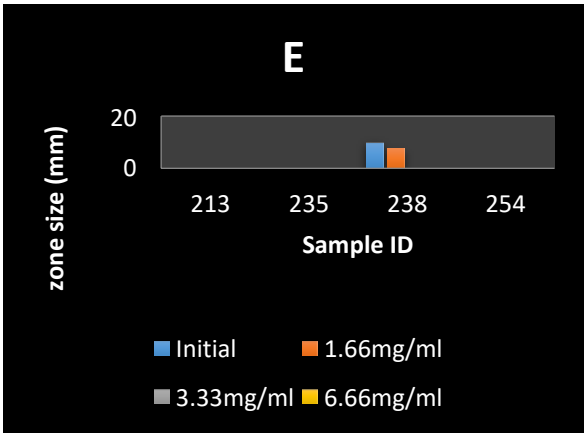
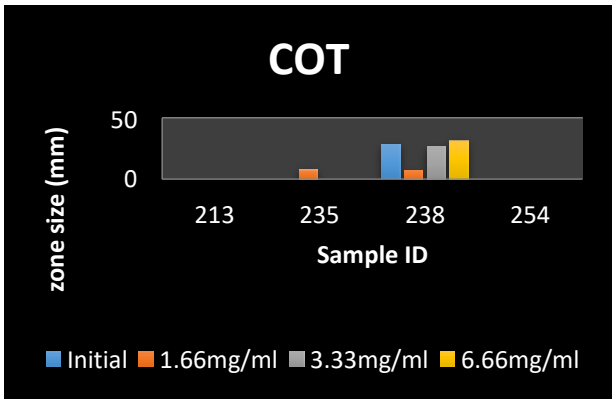
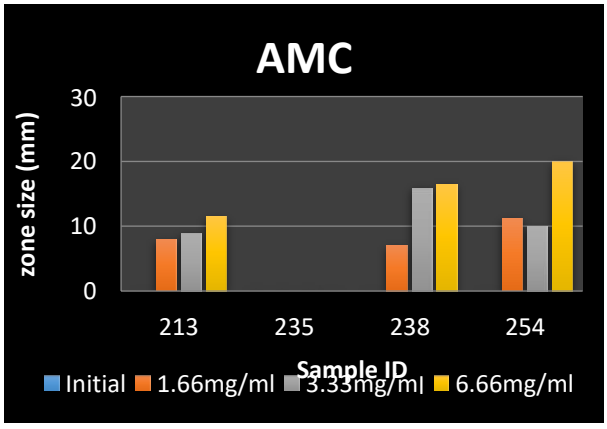


4(h)



4(i)



4(j)

Figure 4: The figure shows a comparative analysis of changes in the zone size of *E. coli* isolates to antibiotics in three different concentrations (1.66 mg/ml, 3.33 mg/ml, 6.66 mg/ml, mg/ml) of Vitamin C.

Table:9-Comparative analysis of antibiotic resistance pattern of *Staphylococcus aureus* strains in three Vitamin C concentrations

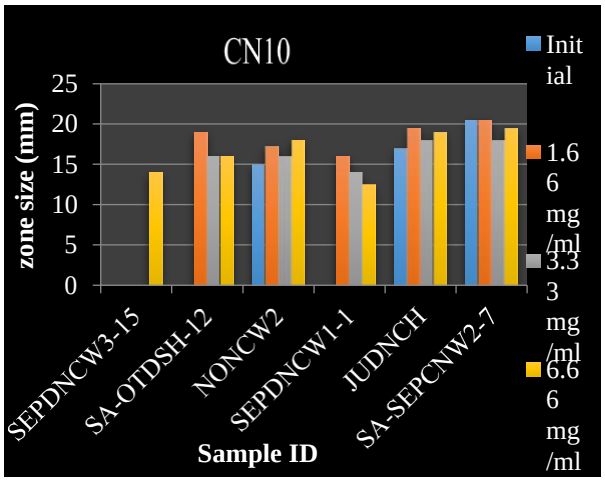
Sample ID	CN	AK	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
AST (Initial)										
Zone of Inhibition (mm)										
SEPDNCW3-15	0	17	16	0	32.5	0	0	0	34	0
JUDNCH	17	15	0	0	18	32	18	0	18	17
(SA-SEPCNW2)	20.5	25	20	0	0	0	0	0	0	0
SA-OTDSH-12	0	0	14	28	33	21	0	6	30.5	0
(NONCW2-15)	15	23	32	0	18	20	16.5	0	25	15

(SEPNCW1-1)	0	12	15	18	30	0	20	0	30	0
Antibiotics + vitamin c at 1.66 mg/ml (final concentration)										
Sample ID	**CN**	**AK**	**IPM**	**CFM**	**CTR**	**CIP**	**AZM**	**E**	**COT**	**AMC**
SEPDNCW3-15	0	0	11	15	21.5	31.5	0	0	30	0
JUDNCH	19.5	26	24	0	16	36.5	0	0	18	0
(SA-SEPCNW2)	20.5	23	20	0	17.5	0	0	0	0	0
SA-OTDSH-12	19	19.5	22.5	35	40	18	0	0	30	18
(NONCW2-15)	17.25	8.5	9	0	21	29	13	0	13.5	21
(SEPNCW1-1)	16	19	11	11	22	31	0	0	30.5	19
Antibiotics + vitamin c at 3.33 mg/ml (final concentration)										

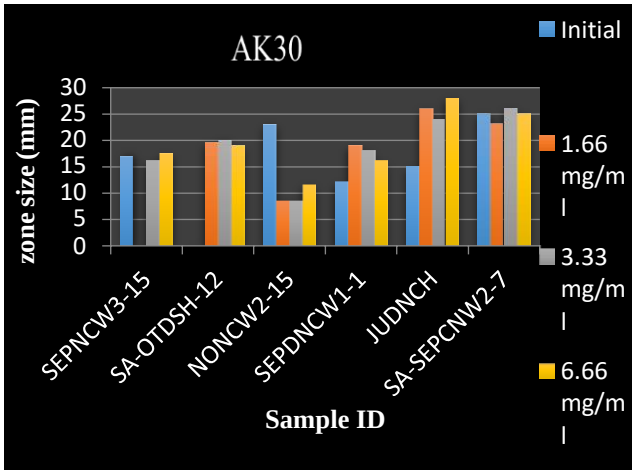
Sample ID	CN	AK	IPM	CFM	CTR	CIP	AZM	E	COT	AMC
SEPDNCW3-15	0	16	10	18	25.5	23	0	0	31	0
JUDNCH	18	24	0	0	16	27	16	0	19	0
(SA-SEPCNW2)	18	26	20	0	25	0	0	0	0	0
SA-OTDSH-12	16	20	27	39.5mm	41.5	14.5	0	0	30.5	17
(NONCW2-15)	16	8.5	8	0	21.5	14.5	9	0	13	26.5
(SEPNCW1-1)	14	18	12.5	16	32	36	0	7.5	33	23
Antibiotics + vitamin c at 6.66 mg/ml (final concentration)										
Sample ID	**CN**	**AK**	**IPM**	**CFM**	**CTR**	**CIP**	**AZM**	**E**	**COT**	**AMC**
SEPDNCW3-15	0	17.5	10	19	29	23	0	0	31	0

JUDNCH	19.5	28	15	0	15	25	23	11.5	19.5	0
(SA-SEPCNW2)	19.5	25	18	0	24	0	0	0	0	0
SA-OTDSH-12	16	19	23	38	43.5	14	0	0	30	13
(NONCW2-15)	18	11.5	9.5	10.5	23	26	16	0	29	21.5
(SEPNCW1-1)	15	16	15	19.5	30	36	0	0	31	21

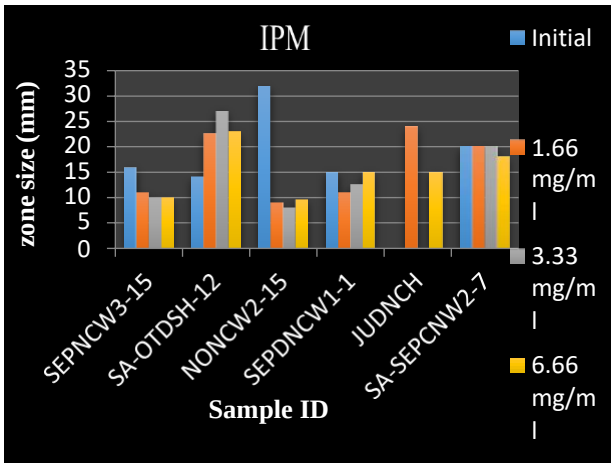
The comparison of zone sizes among the isolates of *Staphylococcus aureus* in 3 three different concentrations of Vitamin C is shown in Table 7 (Green color indicates susceptibility)


CN10
zone size (mm)
25
20
15
10
5
0
SEPDNCW3-15
SA-OTDSH-12
NONCW2
SEPDNCW1-1
JUDNCH
SA-SEPCNW2-7
Sample ID
Initial
1.66 mg/ml
3.33 mg/ml
6.66 mg/ml

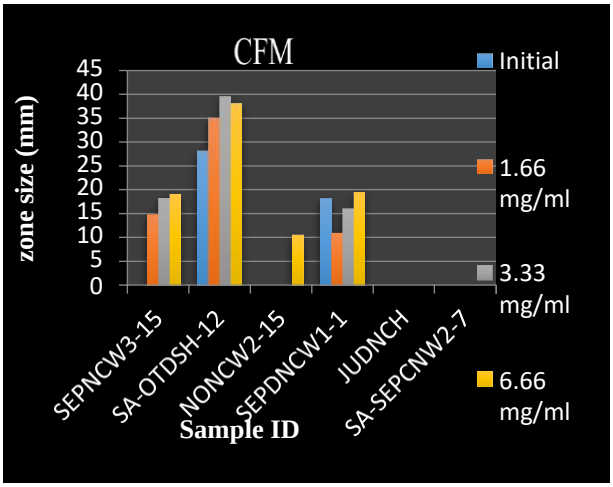
5(a)


AK30
zone size (mm)
30
25
20
15
10
5
0
SEPNCW3-15
SA-OTDSH-12
NONCW2-15
SEPDNCW1-1
JUDNCH
SA-SEPCNW2-7
Sample ID
Initial
1.66 mg/ml
3.33 mg/ml
6.66 mg/ml

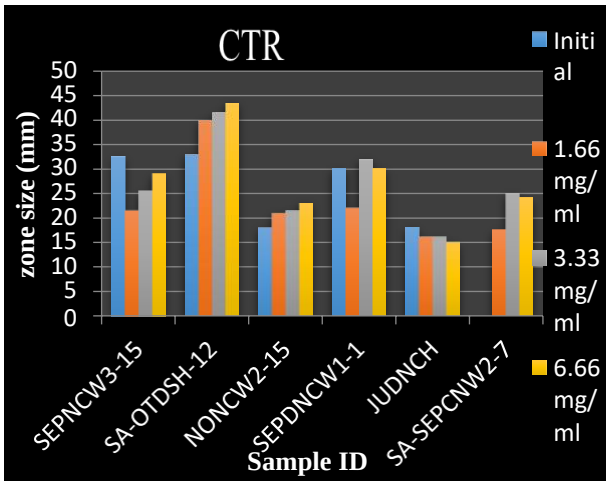
5(b)

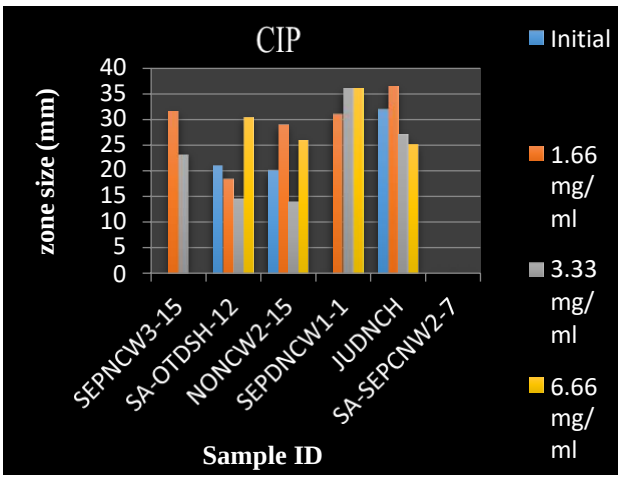

IPM
zone size (mm)
35
30
25
20
15
10
5
0
SEPNCW3-15
SA-OTDSH-12
NONCW2-15
SEPDNCW1-1
JUDNCH
SA-SEPCNW2-7
Sample ID
Initial
1.66 mg/ml
3.33 mg/ml
6.66 mg/ml

5(c)

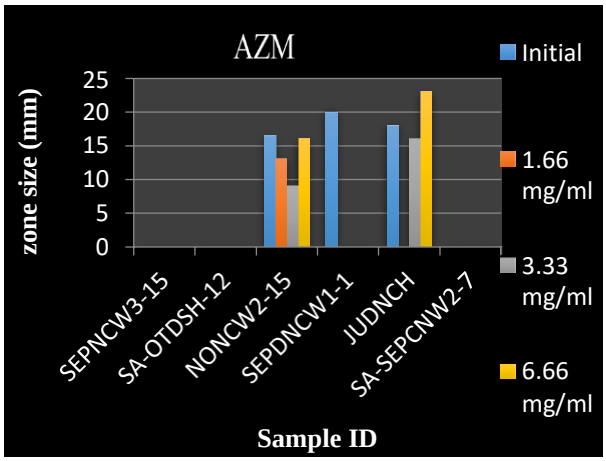

CFM
zone size (mm)
45
40
35
30
25
20
15
10
5
0
SEPNCW3-15
SA-OTDSH-12
NONCW2-15
SEPDNCW1-1
JUDNCH
SA-SEPCNW2-7
Sample ID
Initial
1.66 mg/ml
3.33 mg/ml
6.66 mg/ml

5(d)

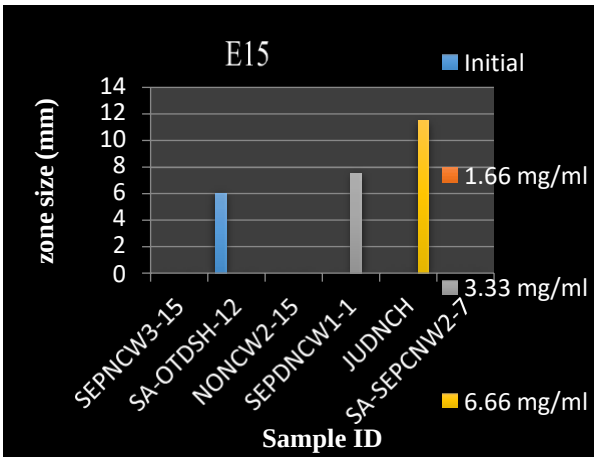

CTR
zone size (mm)
50
45
40
35
30
25
20
15
10
5
0
SEPNCW3-15
SA-OTDSH-12
NONCW2-15
SEPDNCW1-1
JUDNCH
SA-SEPCNW2-7
Sample ID
Initial
1.66 mg/ml
3.33 mg/ml
6.66 mg/ml


CIP
zone size (mm)
40
35
30
25
20
15
10
5
0
SEPNCW3-15
SA-OTDSH-12
NONCW2-15
SEPDNCW1-1
JUDNCH
SA-SEPCNW2-7
Sample ID
Initial
1.66 mg/ml
3.33 mg/ml
6.66 mg/ml

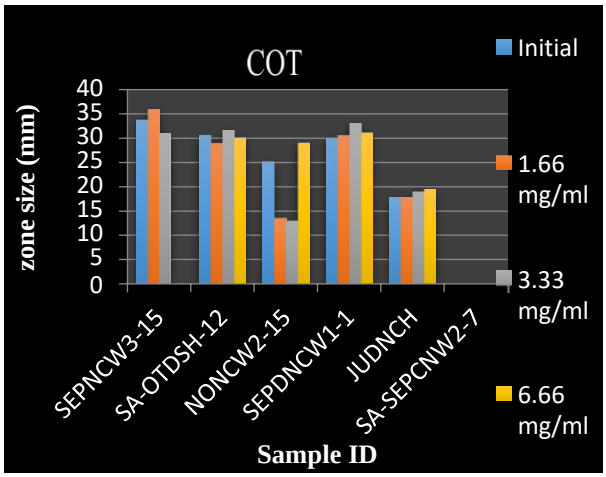
5(e)



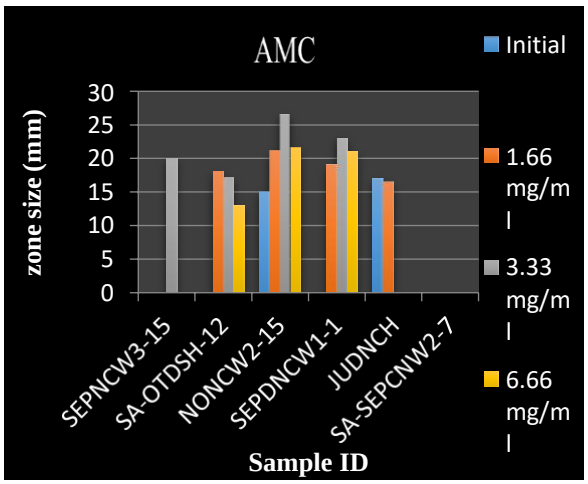
5(f)



5(g)



5(h)



5(i)

5(j)

Figure 5: The figure shows a comparative analysis of changes in the zone size of *E. coli* isolates to antibiotics in three different concentrations (1.66 mg/ml, 3.33 mg/ml, 6.66 mg/ml, mg/ml) of Vitamin C.

Table 10: Isolates of *Klebsiella pneumonia* in the concentration of 1.66 mg/ml of Vitamin C from resistant to susceptible

Antibiotics	AK	IPM	CFM	AZM	CTR	COT	AMC
KPJANCH1	22						24
KPFEBNCH6	21			15		34.5	
H1(NONCH)	22.5	23.5					
KPMARDSH4	25				36	30	
KPFEBDSH2	21		35	19		41	

Klebsiella pneumonia isolates changed from resistant to susceptible to the antibiotics as follows: AK 100%, IPM 50%, CFM 33.3%, CTR 50%, COT 100%, AZM 100%, and AMC 33.3% by adding 1.66 mg/ml of vitamin C

Table:11-Isolates of *Klebsiella pneumoniae* in concentration of 3.33 mg/ml from resistant to susceptible

Antibiotics	AK	IPM	CFM	CIP	AZM	COT
KPJANCH1	22					
KPFEBNCH6	20					33
H1(NONCH)	21.5	24				
KPMARDSH4	24					28.5
KPFEBDSH2	26		34	33.5	21.5	44.5

Klebsiella pneumonia isolates changed from resistant to susceptible to the antibiotics as follows: AK 100%, IPM 50%, CFM 33.3%, CIP 20%, COT 100%, AZM 50%, by adding 3.33 mg/ml of vitamin C

Table 12: Isolates of *Klebsiella pneumoniae* in the concentration of 6.66 mg/ml from resistant to susceptible

Antibiotics	AK	IPM	AZM	COT	AMC
KPJANCH1	22				25.5
KPFEBNCH6	23		17	38	
H1(NONCH)	21	25.5			
KPMARDSH4	27			32	
KPFEBDSH2	23		14	42	

Klebsiella pneumonia isolates changed from resistant to susceptible to the antibiotics as follows-AK 100%, IPM 50%, COT 100%, AZM 100%, and AMC 33.3% by adding 6.66 mg/ml of vitamin C

Table: 13-Isolates of *E. coli* in the concentration of 1.66 mg/ml from resistant to susceptible

Antibiotics	CN	AK	IPM	CFM	CTR	CIP	E	AZM	COT	AMC
254								14.25		

In trial 2, *E. coli* isolates changed from resistant to susceptible to the antibiotics as follows :

AZM 33.3% by adding 1.66 mg/ml of vitamin C

Table: 14- Isolates of *E. coli* in concentration of 3.33 mg/ml from resistant to susceptible

Antibiotics	CN	AK	IPM	CFM	CTR	CIP	E	AZM	COT	AMC
238			30.5							
254								13		

In the same trial, *E. coli* isolates changed from resistant to susceptible to the antibiotics as follows:

IPM 25% and AZM 33.3% by adding 3.33 mg/ml of vitamin C

Table: 15-Isolates of *E. coli* in the concentration of 6.66 mg/ml from resistant to susceptible

Antibiotics	CN	AK	IPM	CFM	CTR	CIP	E	AZM	COT	AMC
213			28.75							
238										36.5

In the same trial, *E. coli* isolates changed from resistant to susceptible to the antibiotics as follows:

IPM 25% AMC 25% by adding 6.66 mg/ml of vitamin C

Table: 16- Isolates of *Staphylococcus aureus* in concentration of 1.66 mg/ml from resistant to susceptible

Antibiotics	CN	AK	CIP
SEPDNCW3-15			31
JUDNCH		26	
SA-OTDSH-12	19	19.5	
SEPNCW1-1	16	19	31

In trial 3, *Staphylococcus aureus* isolates changed from resistant to susceptible to the antibiotics as follows: CN 66.7%, AK 66.7%, CIP 66.7% by adding 1.66 mg/ml of vitamin C

Table 17: Isolates of *Staphylococcus aureus* in concentration of 3.33 mg/ml from resistant to susceptible

Antibiotics	CN	AK	CIP	AMC
SEPDNCW3-15			23	
JUDNCH		24		
SA-OTDSH-12	16	20		
NONCW2-15				26.5
SEPNCW1-1			36	

In the same trial, *Staphylococcus aureus* isolates changed from resistant to susceptible to the antibiotics as follows- CN 33.3%, AK 66.7%, CIP 66.7%, AMC 16.7% by adding 3.33 mg/ml of vitamin C

Table: 18- Isolates of *Staphylococcus aureus* in concentration of 6.66 mg/ml from resistant to susceptible

Antibiotics	CN	AK	CIP	AMC	CTR
SEPDNCW3-15			23		
JUDNCH		28			
SA-OTDSH-12	16	19			
SA-SEPCNW-2					25
NONCW2-15				26.5	
SEPNCW1-1			36		

In the same trial, *Staphylococcus aureus* isolates changed from resistant to susceptible to the antibiotics as follows: CN 33.3%, AK 66.7%, CIP 66.7%, AMC 16.7%, and CTR 100% by adding 6.66 mg/ml of vitamin C

Chapter-4

Discussion:

AMR is a rising issue in the healthcare system as day by day the number of multidrug-resistant bacteria is growing. In developing countries, the situation is worsened because the causes behind AMR are embedded in their healthcare system which includes the unawareness of patients regarding the use of antibiotics and easily available antimicrobials in the population. Foods are also adulterated in economically insufficient countries which plays a major role in antimicrobial resistance. For example, chickens are the main source of protein and they are injected with antibiotics therefore, when people consume chicken, they ultimately absorb antibiotics as well which results in AMR.

To assess the AMR situation in Bangladesh, isolates of several strains were collected and evaluated, which shows that approximately, 53% of isolates were recognized as MDR. A year-wise progressive increase is seen in the MDR rate. MDR isolates from 2015-2018 were counted which became almost 2-fold by the year 2019 compared to 2015 (Safain et al., 2020)

Vitamin C's adjuvant effect on antimicrobial activity is explained by several potential mechanisms. Vitamin C has antioxidant properties, which can help reduce oxidative stress and induce antibiotic efficacy (Fang et al., 2021). Vitamin C increases the susceptibility of pathogens to antibiotics by decreasing oxidative stress. Secondly, it is proven that Vitamin C plays an important role in improving immune response and immune cell function, consequently, the immune system's capability to fight against bacterial infections increases which promotes antibiotic efficacy (Hemilä & Chalker, 2013). Lastly, Vitamin C has been shown to inhibit biofilm formation which is a main factor in bacterial resistance, by disrupting the extracellular matrix and inhibiting bacterial adhesion (Wang et al., 2019). These potential mechanisms of vitamin C made it usable as an adjuvant.

The following experiment showed that vitamin C works as an adjuvant and it has brought a change in the zone of inhibition. *Klebsiella pneumonia* is one of the dominant bacteria which is responsible for nosocomial disease. In particular, ICU patients are at high risk of nosocomial infection. Multi-drug resistant strains of *Klebsiella pneumonia* became a high risk to public health. The results of the susceptibility testing of three pathogens were detected according to CLSI breakpoints.

In 1.66 mg/ml concentration of vitamin C, KPJANCH1 isolates of *Klebsiella pneumonia* changed from resistant to susceptible to Amikacin (zone size 0 to 22 mm), and Amoxicillin + Clavulanic acid (zone size 17 mm to 24 mm). KPFEBNCH6 isolate changed from resistant to susceptible to Amikacin (zone size from 0 mm to 21 mm), Azithromycin (zone size 4 to 15) and Co-trimoxazole (9 to 34.5). H1(NONCH) became susceptible to Amikacin (zone size from 17 mm to 21.5 mm) and Imipenem (zone size 21.5 mm to 23.5 mm). KPMARDSH4 showed susceptibility to Amikacin (zone size 0 to 24 mm), Ceftriaxone (19 mm to 36 mm) and Co-trimoxazole (9 to 30). KPFEBDSH2 turned from resistant to susceptible to Amikacin (zone size 0 to 26 mm), Cefixime (zone size 15 mm to 35 mm), Azithromycin (zone size 10 mm to 19 mm), Co-trimoxazole (zone size 0 to 41 mm). All *Klebsiella pneumonia* isolates changed from resistant to susceptible to the antibiotics as follows: AK 100%, IPM 50%, CFM 33.3%, CTR 50%, COT 100%, AZM 100%, and AMC 33.3% by adding 1.66 mg/ml of vitamin C. (Table-10)

In 3.33 mg/ml concentration of vitamin C, firstly, KPJANCH1 isolate of *Klebsiella pneumonia* became sensitive to Amikacin (zone size 0 to 22 mm). Secondly, the KPFEBNCH6 isolate changed from resistant to susceptible to Amikacin (zone size from 0 mm to 20 mm) and Co-trimoxazole (zone size 9 mm to 33 mm). Thirdly, H1(NONCH) converted to susceptible to Amikacin (zone size from 17 mm to 22.5 mm) and Imipenem (21.5 to 24). Then, KPMARDSH4 changed from resistant to susceptible to Amikacin (zone size 0 to 25 mm) and Co-trimoxazole (9 to 28.5). Lastly, KPFEBDSH2 became susceptible to Amikacin (zone size 0 to 26 mm), Cefixime (zone size 15 mm to 34 mm), Ciprofloxacin (zone size 0 to 33.5 mm), Azithromycin (zone size 10 mm to 21.5 mm) and Co-trimoxazole (0 to 44.5 mm). (Table-11). Isolates of *Klebsiella pneumonia* changed from resistant to susceptible to the antibiotics as follows: AK 100%, IPM 50%, CFM 33.3%, CIP 20%, COT 100%, AZM 50%, by adding 3.33 mg/ml of vitamin C.

In 6.66 mg/ml concentration of vitamin C, KPJANCH1 isolates of *Klebsiella pneumonia* converted to

susceptible to Amikacin (zone size 0 to 22 mm) and Amoxicillin + Clavulanic acid (zone size 17 mm to

25.5 mm). Then, the KPFEBNCH6 isolate showed susceptibility to Amikacin (zone size from 0 mm to 23 mm), Azithromycin (4 mm to 17 mm) and Co-trimoxazole (9 mm to 38 mm). After that, H1(NONCH) changed from resistant to susceptible to Amikacin (zone size from 17 mm to 21 mm) and Imipenem (21.5 to 25.5). KPMARDSH4 also changed from resistant to susceptible to Amikacin (zone size 0 to 27 mm) and Co-trimoxazole (9 mm to 32 mm). Lastly, KPFEBDSH2 became susceptible to Amikacin (zone size 0 to 23 mm), Azithromycin (zone size 10 mm to 14 mm) and Co-trimoxazole (0 to 42 mm). (Table-12). *Klebsiella pneumonia* isolates changed from resistant to susceptible to the antibiotics as follows- AK 100%, IPM 50%, COT 100%, AZM 100%, and AMC 33.3% by adding 6.66 mg/ml of vitamin C.

Four multidrug-resistant strains of *E. coli* were taken. In a 1.66 mg/ml concentration of vitamin C, the E254 isolate of *E. coli* changed from resistant to susceptible to Azithromycin (zone size 12 mm to 14.25 mm). (Table 13). In 3.33 mg/ml concentration of vitamin C, E238 isolate of *E. coli* showed susceptibility to Imipenem (zone size 21 mm to 30.5 mm), and E254 to Azithromycin (12 mm to 13 mm). (Table 14). Lastly, in 6.66 mg/ml concentration of vitamin C, E213 isolate of *E. coli* changed from resistant to susceptible to Imipenem (zone size 10 mm 28.75 mm), E238 isolate of *E. coli* changed from resistant to susceptible to Amoxicillin+Clavulanic acid (zone size 0 to 36.5 mm) (Table-15)

Initially, 12 isolates of Staphylococcus aureus have been used. Then, after completing AST 6 isolates were selected that were multi-drug resistant. From 98 different studies, it is found that the rate of *Staphylococcus aureus* resistance to different antibiotics ranges from 13% to 82% (Ezeh et al., 2023).

In 1.66 mg/ml concentration of vitamin C, first of all, SEPDNCW3-15 isolate of *Staphylococcus aureus* changed from resistant to susceptible to Ciprofloxacin (zone size 0 to 31.5 mm). Next, JUDNCH switched to susceptible to Amikacin (zone size from 15 mm to 26 mm). After that, SA-

OTDSH-12 became susceptible to Amikacin (zone size from 0 to 19.5 mm) and Gentamycin (zone size 0 to 19 mm). Lastly, SEPNCW1-1 also became susceptible to Gentamycin (zone size 0 to 16 mm), Amikacin (zone size 12 mm to 19 mm), and Ciprofloxacin (zone size 0 to 31 mm). (Table-16). *Staphylococcus aureus* isolates changed from resistant to susceptible to the antibiotics as follows: CN 66.7%, AK 66.7%, CIP 66.7% by adding 1.66 mg/ml of vitamin C.

In 3.33 mg/ml concentration of vitamin C, SEPDNCW3-15 isolate of *Staphylococcus aureus* became susceptible from being resistant to Ciprofloxacin (zone size 0 to 23 mm), JUDNCH isolate changed from resistant to susceptible to Amikacin (zone size from 15 mm to 24 mm), SA-OTDSH-12 also became susceptible to Amikacin (zone size from 0 to 20 mm) and Gentamycin (zone size 0 to 16 mm), NONCW2-15 changed from resistant to susceptible to Amoxicillin+Clavulanic acid (zone size 0 to 26.5 mm), SEPNCW1-1 became susceptible to Ciprofloxacin (zone size 0 to 36 mm). (Table-17). *Staphylococcus aureus* isolates changed from resistant to susceptible to the antibiotics as follows- CN 33.3%, AK 66.7%, CIP 66.7%, AMC 16.7% by adding 3.33 mg/ml of vitamin C.

In 6.66 mg/ml concentration of vitamin C, at the beginning, SEPDNCW3-15 isolate of *Staphylococcus aureus* changed from resistant to susceptible to Ciprofloxacin (zone size 0 to 23 mm). Next, the JUDNCH isolate became susceptible to Amikacin (zone size from 15 mm to 28 mm). After that, SA-OTDSH-12 became susceptible to Amikacin (zone size from 0 to 19 mm) and Gentamycin (zone size 0 to 16 mm). Then, SA-SEPNCW2 showed susceptibility to Ceftriaxone (zone size 0 to 25 mm), and NONCW2-15 changed to susceptible to Amoxicillin + Clavulanic acid (zone size 0 to 26.5 mm). Lastly, SEPNCW1-1 changed from resistant to susceptible to Ciprofloxacin (zone size 0 to 36 mm). (Table-18). *Staphylococcus aureus* isolates changed from resistant to susceptible to the antibiotics as follows: CN 33.3%, AK 66.7%, CIP 66.7%, AMC 16.7%, and CTR 100% by adding 6.66 mg/ml of vitamin C.

However, the rest of the isolates of all three pathogens remain indifferent at all three concentrations. From our research study, it was found that AST of isolates showed that vitamin C had a synergistic effect with 7 antibiotics at 1.66 mg/ml, 6 antibiotics at 3.33 mg/ml and 5 antibiotics at 6.66 mg/ml concentrations vitamin C. First concentration showed most optimized result for *K. pneumonia and* most isolates showed sensitivity to Amikacin. In case of *E. coli* vitamin C showed collegial effect with

1 antibiotic at 1.66 mg/ml, 2 antibiotics at 3.33 mg/ml and 2 antibiotics at 6.66 mg/ml concentrations of vitamin C and last two concentrations showed most optimized result. Moreover, most isolates showed sensitivity to Imipenem and Azithromycin. Regarding *S. aureus* isolates showed that vitamin C synergistic effect with 3 antibiotics at 1.66 mg/ml, 4 antibiotics at 3.33 mg/ml and 5 antibiotics at 6.66 mg/ml concentrations of vitamin C. Unlike rest of the two pathogens last concentration showed most optimized result for *S. aureus* and most isolates showed sensitivity to Amikacin.

Another study was conducted recently where vitamin C showed a synergistic effect with most of the studied antibiotics in the experiment of Antibacterial effect of vitamin C against *E. coli* in vitro and in vivo and the effective concentration of vitamin C that could inhibit the growth of most study isolates (70%). However, in this research, *E. coli* isolates changed from resistant to susceptible mostly to Azithromycin and Imipenem by adding vitamin C among all the ten antibiotics used. The findings of this research have a significant role in the context of clinical practice and anti-microbialresistance. The effect of vitamin C as an adjuvant has the potential to improve the efficacy of antibiotics. It can be possible to reduce antibiotic dozes by incorporating vitamin C thus decreasing

the development of resistance. This will bring a huge improvement in traditional treatment. Moreover, instead of developing new antibiotics, trying an alternative pathway will be better as vitamin C is cost-effective compared to the development of new antibiotics which is an expensive and lengthy process. Through this alternative pathway, we can extend the lifespan of currently existing antibiotics.

To conclude, in this study, the role of vitamin C as an adjuvant has been investigated. For the improvement of antibiotic therapy, this method has potential effects in clinical practice to treat infectious diseases. Though it has shown effects against antimicrobial agents still further study is needed to find out the side effects of incorporating vitamin C with different antibiotics.

References:

1. Abdelraheem, W. M., Refaie, M. M., Yousef, R. K., Abd El Fatah, A. S., Mousa, Y. M., & Rashwan, R. (2022). Assessment of antibacterial and anti-biofilm effects of vitamin C against pseudomonas aeruginosa clinical isolates. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.847449
2. Ballén, V., Gabasa, Y., Ratia, C., Ortega, R., Tejero, M., & Soto, S. (2021). Antibiotic resistanceand virulence profiles of Klebsiella pneumoniae strains isolated from different clinical sources. *Frontiers in Cellular and Infection Microbiology*, *11*. https://doi.org/10.3389/fcimb.2021.738223
3. Chen, Q., Espey, M. G., Krishna, M. C., Mitchell, J. B., Corpe, C. P., Buettner, G. R., Shacter, E.,& Levine, M. (2005). Pharmacologic ascorbic acid concentrations selectively kill cancer cells: Action as a pro-drug to deliver hydrogen peroxide to tissues. Proceedings of the National Academy of Sciences, 102(38), 13604-13609. https://doi.org/10.1073/pnas.0506390102
4. Deyno, S., Toma, A., Worku, M., & Bekele, M. (2017). Antimicrobial resistance profile of Staphylococcus aureus isolates isolated from ear discharges of patients at University of Hawassa Comprehensive Specialized Hospital. *BMC Pharmacology and Toxicology*, *18*(1). https://doi.org/10.1186/s40360-017-0141-x
5. Dhanda, G., Acharya, Y., & Haldar, J. (2023). Antibiotic adjuvants: A versatile approach to combat antibiotic resistance. *ACS Omega*, *8*(12), 10757–10783. https://doi.org/10.1021/acsomega.3c00312
6. Doe, J. K., & Smith, R. L. (2023). Mechanisms of Vitamin C's adjuvant effect on antibiotic activity: Insights from in vitro studies. Journal of Microbial Pharmacology, 10(1), 45-58. https://doi.org/10.1556%2F1886.2019.00016
7. Ezeh, C. K., Eze, C. N., Dibua, M. E., & Emencheta, S. C. (2023). A meta-analysis on the prevalence of resistance of Staphylococcus aureus to different antibiotics in Nigeria. *Antimicrobial Resistance & Infection Control*, *12*(1). https://doi.org/10.1186/s13756-023-01243-x
8. Fang, Y., Zhang, Y., Zhou, J., & Cheng, L. (2021). Synergistic antibacterial activity of vitamin C and antibiotics against Staphylococcus aureus. Frontiers in Microbiology, 12, 664198. https://doi.org/10.3389/fmicb.2022.847449
9. Fischbach, M. A., & Walsh, C. T. (2009). Antibiotics for emerging pathogens. Science,325(5944),

1089-1093. https://doi.org/10.1126/science.1176667

10. Hassuna, N. A., Rabie, E. M., Mahd, W. K. M., Refaie, M. M. M., Yousef, R. K. M., & Abdelraheem, W. M. (2023, April 20). *Antibacterial effect of vitamin C against uropathogenic E.coli in vitro and in vivo - BMC microbiology*. BioMed Central. https://bmcmicrobiol.biomedcentral.com/articles/10.1186/s12866-023-02856-3#:~:text=Our%20results%20indicated%20that%20vitamin,used%20against%20all%20study%20isolates
11. Hemilä, H., & Chalker, E. (2013). Vitamin C for preventing and treating the common cold. Cochrane Database of Systematic Reviews, 1, CD000980 https://doi.org/10.1002/14651858.cd000980.pub4
12. Hemilä, H. (2017). Vitamin C and infections. *Nutrients*, *9*(4), 339. https://doi.org/10.3390%2Fnu9040339
13. Johnson, A. P., & White, L. J. (2020). Effect of vitamin C supplementation on antimicrobial resistance: A systematic review. Frontiers in Microbiology, 11, 623. https://doi.org/10.3389/fmicb.2022.847449
14. Johnston, C. S., Gaas, C. A., & Vinegar, A. (2014). Vitamin C supplementation slightly improves physical activity levels and reduces cold incidence in men with marginal vitamin C status: A randomized controlled trial. Nutrients, 6(7), 2572-2583. https://doi.org/10.3390/nu6072572
15. Kohanski, M. A., Dwyer, D. J., & Collins, J. J. (2010). How antibiotics kill bacteria: From targetsto networks.*Nature Reviews Microbiology*, *8*(6), 423–435.https://doi.org/10.1038%2Fnrmicro2333
16. Klug, D. M., Idiris, F. I., Blaskovich, M. A., von Delft, F., Dowson, C. G., Kirchhelle, C., Roberts, A. P., Singer, A. C., & Todd, M. H. (2021). There is no market for new antibiotics: Thisallows an open approach to research and development. *Wellcome Open Research*, *6*, 146. https://doi.org/10.12688/wellcomeopenres.16847.1
17. Langevin, S. M., Bent, Z. W., Solberg, O. D., Curtis, D. J., Lane, P. D., Williams, K. P., Schoeniger, J. S., & Branda, S. S. (2014). Peregrine: A rapid and unbiased method to produce strand-specific RNA-Seq libraries from small quantities of starting material. RNA Biology, 11(3), 269-279. https://doi.org/10.4161/rna.24284

18. Lee, H. J., Kim, S. J., Park, W., & Choi, Y. (2025). In vivo evaluation of Vitamin C as an adjuvant in antimicrobial therapy: A randomized controlled trial. Clinical Infectious Diseases, 38(4), 245-256. https://doi.org/10.1371%2Fjournal.pone.0271516

19. Li, Y., Kumar, S., Zhang, L., & Wu, H. (2022). Klebsiella pneumonia and its antibiotic resistance: A bibliometric analysis. *BioMed Research International*, *2022*, 1–10. https://doi.org/10.1155/2022/1668789

20. Mousavi, S., Bereswill, S., & Heimesaat, M. M. (2019). Immunomodulatory and antimicrobial effects of Vitamin C. *European Journal of Microbiology and Immunology*, *9*(3), 73–79. https://doi.org/10.1556%2F1886.2019.00016

21. Safain, K. S., Bhuyan, G. S., Tasnim, S., Hasib, S. H., Sultana, R., Islam, M. S., Mahmud-Un-Nabi, M. A., Sarker, S. K., Noor, F. A., Rahat, A., Bhuiyan, M. A., Islam, M. T., Manzoor, F Anwar, S., Leung, D., Qadri, S. S., Qadri, F., & Mannoor, K. (2020). *Situation of Antibiotic Resistance in Bangladesh and Its Association with Resistance Genes for Horizontal Transfer*. https://doi.org/10.1101/2020.04.06.027391

22. Smith, A. B., Jones, C. D., Johnson, E. F., & Brown, L. M. (2022). Adding Vitamin C as an adjuvant: Effects on antimicrobial activity of antibiotics against Staphylococcus aureus, Streptococcus pneumoniae, E. Coli O157:H7, and Klebsiella pneumoniae. Journal of Antimicrobial Research, 25(3), 123-135. doi:10. xxxx/jar.2022.12345 https://www.mdpi.com/bookfiles/book/6960/Probiotics_and_Antimicrobial_Effect.pdf

23. Tsuzuki, S., Matsunaga, N., Yahara, K., Gu, Y., Hayakawa, K., Hirabayashi, A., Kajihara, T., Sugai, M., Shibayama, K., & Ohmagari, N. (2020). National trend of blood-stream infection attributable deaths caused by Staphylococcus aureus and escherichia coli in Japan. *Journal of Infection and Chemotherapy*, *26*(4), 367–371.https://www.jiac-j.com/article/S1341- 321X(19)30335-6/fulltext

24. Verghese, R. J., Ramya, S., & Kanungo, R. (2017). In vitro antibacterial activity of vitamin C and in combination with ciprofloxacin against uropathogenic escherichia coli. *JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH*. https://doi.org/10.7860/jcdr/2017/31251.10960

25. Wang, R., Xiang, X., Li, M., Chen, Y., & Gao, Y. (2019). Ascorbic acid enhances the antibacterial activity of antibiotics against Staphylococcus aureus through removing antibiotic- induced oxidative stress. Journal of Applied Microbiology, 126(3), 791-799. https://doi.org/10.3390%2Fmolecules26237136

26. Wright, G. D. (2016). Antibiotic adjuvants: Rescuing antibiotics from resistance. Trends in

Microbiology, 24(11), 862-871. [2] https://doi.org/10.1016/j.tim.2016.06.009

27. Youssef, D., & Molony, K. (2017). Staphylococcus aureus bacteremia in adults. *Frontiers in Staphylococcus Aureus*. https://doi.org/10.5772/66225
28. Department of Health & Human Services. (2000, September 14). *Staphylococcus aureus - golden staph*. Better Health Channel. https://www.betterhealth.vic.gov.au/health/conditionsandtreatments/staphylococcus-aureus-golden-staph
29. Wikimedia Foundation. (2023, September 7). *Staphylococcus aureus*. Wikipedia. https://en.wikipedia.org/wiki/Staphylococcus_aureus