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RESEARCH ARTICLE
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EVALUATION OF SYNERGISTIC EFFECT OF SYNTHETIC DRUGS WITH VITAMINS ON MULTIDRUG RESISTANT BACTERIA ISOLATED FROM URINARY TRACT INFECTION

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ABSTRACT

Urinary tract infections (UTI's) are the most common bacterial infections experienced by patients. These infections are often asymptomatic, but sometimes they produce discomfort for selective older patients, and present a risk for bacteremia, septic shock, adult respiratory distress syndrome, and are rarely fatal. Antibiotics are a powerful line of defense against bacterial infections. Frequent use of antibiotics can drastically change the amount and types of bacteria within the gut microbiota especially in early life. Antibiotics are important for treating infections. However, if overused, they can cause long-term changes to healthy gut bacteria and contribute to liver damage, which can lead to antibiotic-associated diseases, especially in children. Fortunately, a number of studies have shown that taking vitamin can reduce the risk of antibiotic-associated diseases. In the current study, total 100 urine samples were collected from UTI patients. Out of the collected samples the proportion of extended spectrum beta lactamase *Klebsiella pneumonia,* Methicillin resistant *Staphylococcus aureus* and aminoglycoside resistant *Enterococcus faecalis* was found to be 48%, 30% and 22% respectively. Standard microbial techniques like bacterial culture and several biochemical tests were applied for bacterial isolation and identification. Methicillin resistant *Staphylococcus aureus* initially known to be resistant against cefoxitin was found vulnerable when a combination of cefoxitin and vitamin B6 (10ug/ul) was added to the bacterial culture which showed an inhibition zone of 24mm. It was concluded that the efficacy of synergism of synthetic antibiotics and vitamins was assessed as a promising antibacterial approach for the

INTRODUCTION

Antibiotics have long served as the cornerstone for treating bacterial infections. However, their overuse particularly during early developmental stages—has led to significant disruptions in gut microbiota composition. This can cause long-term consequences, including liver damage and an increased susceptibility to antibiotic-associated diseases, especially in children. Studies have shown

that probiotics, particularly *Lactobacilli* and *Saccharomyces* strains, can help restore healthy microbiota after antibiotic treatment and reduce the risk of associated complications (Robertson et al., 2018). Probiotics are generally defined as microbial food supplements that offer a range of health benefits, such as improving intestinal health, enhancing immunity, increasing nutrient bioavailability, and reducing the risk of certain cancers (Parvez et al., 2006).

One of the primary pathogens of concern in antibiotic-resistant infections is *Klebsiella pneumoniae*. This Gram-negative, non-motile, encapsulated bacillus is part of the Enterobacteriaceae family and typically resides harmlessly in the gastrointestinal tract. However, if translocated to other parts of the body, it can cause severe infections such as pneumonia, liver abscesses, septicemia, and urinary tract infections (UTIs) (Paczosa & Mecsas, 2016). *K. pneumoniae* is a common cause of UTIs, particularly in elderly individuals and catheterized patients. It has shown increasing resistance to antibiotics through the production of extended-spectrum beta-lactamases (ESBLs) and carbapenemases such as NDM-1, rendering many antibiotics ineffective (Murugan & Paulpandian, 2013).

The global dissemination of carbapenem-resistant *K. pneumoniae*, especially sequence type ST258 carrying KPC (Klebsiella pneumoniae carbapenemase) genes, underscores the serious public health threat posed by this organism (Grashorn, 2010; Daikos et al., 2014). These resistance genes are often located on transposable elements like Tn4401, facilitating horizontal gene transfer. Consequently, *K. pneumoniae* exhibits both high virulence and environmental persistence, with resistance mechanisms that include altered membrane permeability and synergistic action with other β-lactamases (Sbrana et al., 2012; Elemam et al., 2010).

Another major MDR pathogen is *Staphylococcus aureus*, particularly its methicillin-resistant strain (MRSA), which is implicated in various infections ranging from skin abscesses to pneumonia and endocarditis. MRSA is resistant to almost all β-lactam antibiotics due to the mecA gene, which encodes an altered penicillin-binding protein (PBP2a) (Chambers & DeLeo, 2009). These bacteria are capable of producing a range of virulence factors such as protein A, PVL (Panton-Valentine leukocidin), and superantigens, which facilitate immune evasion and tissue damage (Peacock et al., 2019; Villegas-Estrada et al., 2008). The increasing prevalence of MRSA strains in both hospital and community settings is attributed to overuse and misuse of antibiotics, which exert selective pressure on bacterial populations. The failure to develop effective vaccines further complicates the management of MRSA infections. Furthermore, even once-effective antibiotics like aminoglycosides have become less reliable due to evolving resistance mechanisms that disrupt ribosomal binding (Van Bambeke et al., 2008).

Streptococcus pyogenes, or Group A Streptococcus (GAS), is another relevant pathogen. It causes a spectrum of diseases including pharyngitis, impetigo, and toxic shock-like syndrome. GAS expresses various virulence factors such as M proteins, streptolysins, hyaluronidase, and DNases that contribute to its pathogenicity (Albrich et al., 2004). New evidence suggests that vitamin D derivatives like doxercalciferol exhibit antimicrobial properties against *Streptococcus* species and may work synergistically with antibiotics such as bacitracin by interfering with cell wall synthesis (Gyll et al., 2018). Interestingly, antibiotics themselves can have complex effects on bacterial protein expression. For example, clindamycin (CLDM) has been shown to both suppress and induce the expression of specific streptococcal exotoxins, depending on the concentration and exposure time (Seppälä et al., 1992). The interplay between antibiotics, probiotics, and vitamins is becoming an increasingly important area of investigation in the fight against MDR pathogens. Probiotics not only help maintain healthy gut flora disrupted by antibiotics but also modulate the host immune system by producing antimicrobial compounds and competing for binding sites (LeBlanc et al., 2010). When used in conjunction with vitamins—such as vitamin C, which enhances neutrophil function and increases antibody production—the combination has shown potential in enhancing immune response during infection (LeBlanc et al., 2017).

The role of vitamins in infection control extends beyond immune support. Observational studies have indicated that high intake of antioxidant vitamins correlates with a reduced risk of chronic diseases and may offer protective effects against microbial infections. Vitamin D, in particular, has gained attention for its immunomodulatory and potential antimicrobial properties. However, randomized controlled trials have produced mixed results, and high-dose supplementation may, in some cases, be associated with adverse outcomes (LeBlanc et al., 2017).

In the context of urinary tract infections, which are commonly treated with antibiotics like fluoroquinolones, disruption of normal urinary and gut microbiota can predispose patients to recurrent infections. Thus, supplementing antibiotic therapy with probiotics and vitamins may represent a holistic approach that addresses both pathogen eradication and host microbiome preservation.

Given the alarming rise in multidrug-resistant organisms such as *Klebsiella pneumoniae*, MRSA, *Streptococcus pyogenes*, and *E. coli*, there is an urgent need to explore adjunct strategies that enhance antibiotic efficacy and limit resistance development. The aim of this study is to investigate whether the combination of synthetic antibiotics with vitamins and probiotics results in a synergistic effect against MDR pathogens isolated from urinary tract infections. Specifically, this study seeks to determine: (1) whether vitamins and probiotics act as effective adjuncts to antibiotics, (2) the predominant UTI-causing pathogens such as *K. pneumoniae*, MRSA, *E. faecalis*, and *E. coli*, (3) the antibiotic sensitivity profiles of these isolates, and (4) the efficacy of antibiotic–vitamin combinations in inhibiting or eliminating these resistant bacterial strains.

MATERIALS & METHODS

Inclusion Criteria

Urinary tract infected patients containing pathogens i.e. extended-spectrum β-lactamase *Klebsiella pneumonia,* Methicillin-resistant *Staphylococcus aureus* (MRSA) and high-level aminoglycoside resistant *Enterococcus faecalis* were included in this study.

Exclusion Criteria

Sample of patients other than urinary tract infection were excluded in this study.

Sample Collection

A total 100 samples of urine were collected of those patients, infected with urinary tract infection from the different diagnostic labs and tested performed at Chagatai's Laboratory, Lahore MRSA, *K. pneumonae* and *E. faecalis* were isolated by different nutritional agar media like cysteine lactose electrolyte deficient agar (CLED), MacConkey agar and blood agar.

Isolation of Gram Positive Bacteria Through Culture Technique

MRSA and *E. faecalis* urine specimens were inoculated on the blood agar plate. After streaking, the plate was incubated at 37ºC for 24 hrs. After 24 hrs, growth has been checked and bacteria were isolated through colony morphology. Classic Gram staining technique was performed for Gram –ve and Gram +ve bacteria via following:

Bacterial colony was picked and made a smear on a frosted glass slide then slide was fixed either by heating or by using methanol. Primary stain (crystal violet) applied for 10 secs and rinsed. Mordant iodine solution was added that formed a crystal violet-iodine (CV-I) complex. In decolourization step, the organic solvent (acetone or ethanol) was added for 5-10 sec that extracted the blue dye complex from the lipid-rich, thin-walled Gram-negative bacteria to a greater degree than from the lipid-poor, thick-walled, Gram-positive bacteria. In last step, counterstain (safranin) applied on smear for 15 secs and rinsed.

Isolation of K. pneumonae

Urine samples were inoculated on MacConkey agar to isolate extended *K. pneumonaea.* Then it was incubated at 37ºC for 24 hours. After that incubation for 24 hours, bacteria were isolated based on colonial morphology, along with string method test.

String Method

Clean grease-free slide was taken and put a drop of 0.5% bile salt. The isolated colony of the bacterium was emulsified using inoculating loop. The rubbing was continued with loop vigorously for 2-3 mints to get its vicious appearance. Lastly, the loop was pulled upward from the slide.

Biochemical Identification of Bacteria

Biochemical characterization of bacterial and fungal species was performed using api20 E in which 20 different tests were included (Hussain et al., 2013). For the identification of *Enterobacteriaceae* Gram-ve rods, selected colony was mixed in 5 ml of distilled water ampule and respective wells were filled with 0.5 ml of suspension in api20 E in each cupule, slightly under fill ADH, LDC, ODC, H_2S And URE and completely fill cupules of CIT, VP and GEL, anaerobic condition were created for ADH, LDC, ODC, H_2S, and URE by overlying with mineral oil, after filling all wells of api20 E incubated at 37°c for 24 hours.

Identification of MRSA

Catalase test was performed to differentiate *staphylococcus. aureus* from other Gram positive cocci. *S. aureus* is catalase positive. In this test, a drop of hydrogen peroxide (3%) was placed on glass slide cardboard and a single isolated colony was mixed in this drop. Bubbles appeared that indicated the presence of enzyme catalase while catalase negative bacteria gave no reaction (Bennett et al., 1986).

Coagulase Test

S. aureus was further confirmed by a positive bond coagulase test, which converted fibrinogen directly to fibrin. For that purpose, 1 ml of bacterial suspension was mixed in 200ul of human plasma in a tube and incubated at 37ºC for 24 hours. Plasma was clotted by converted fibrinogen to fibrin, due to the presence of coagulase enzyme which produced by *S. aureus*.

Antibacterial Resistance Assessments of Synthetic Antibiotics

Antibacterial activity of antibiotics gentamicin(120µg), cefoxitin was checked against Gram positive bacteria and for Gram negative bacteria amoxicillin (AMC), cetirizine (CAZ), ceftazidime (CAZ), FEP cefipime via Kerby Bauer method (Balouiri et al., 2015). Muller hinton agar media (Oxoid) was used to check the antimicrobial susceptibility testing (AST) against MRSA. Media was prepared, autoclaved and poured 20 ml in the petri dishes, with depth kept 4mm. Bacterial suspension of 0.5 McFarland was prepared in autoclaved normal saline. With swab stick suspension of bacterial strain was lawned and synthetic antibiotics was applied on plates, then plates were kept in an incubator at 37ºC for 24 hours. The zone of inhibition was measured with a transparent ruler from the back of the plate; also measured the diameter of the zone of inhibition included the diameter of the disc.

Antibacterial Activity Assessments of Different Vitamins

Antibacterial activity of vitamins was checked against MRSA, *E. faecalis* and *K. pneumonae* via a well plate method (Balouiri et al., 2015). Blood agar media (Oxoid) was used to check the antimicrobial susceptibility testing (AST) against MRSA *E. faecalis*. Muller hinton agar media was used for antimicrobial susceptibility testing (AST) against *K. pneumonae*.

Media prepared autoclaved and poured 20 ml in the petri dishes, depth of 4mm. Bacterial suspension of 0.5 MacFarland prepared in autoclaved normal saline. After solidifying the media, lawned with sterilized cotton swabs. In addition, after that wells prepared of 6mm, with the help of 1ml tips and

then plates kept at room temperature for 10-15 minutes. 100 µl volumes of vitamins (B1, B2 and B6) with concentration of 10mg/ml applied in the wells along with negative control. Each experiment performed in triplicate for confirmation.

Synergistic Effect of Vitamins with Synthetic Antibiotics

The procedure was the same as mentioned above. In this step after wells was prepared, synthetic antibiotics with vitamins applied in combination. Inoculated cultured plates incubated at 37°C for 24 hrs. After 24 hrs, zone of inhibition measured. Minimal inhibitory concentration also measured via agar well diffusion method. This provided rapid results of the antimicrobial agent's effects and a better understanding of their impact on the viability and cell damage inflicted on the tested microorganism. The agar plate surface then inoculated by spreading a volume of the microbial inoculum over the entire agar surface. A hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip (well method technique), and a volume (20–100 µL) of the antimicrobial agent or extracted solution at desired concentration, introduced into the well. Then, agar plates incubated under suitable conditions depending upon the test microorganism. The antimicrobial agents diffused in the agar medium and inhibit the growth of the microbial strain tested.

Susceptibility to Antibiotics using the Agar Diffusion Method

The agar diffusion assay was used for quantifying the ability of antibiotics to inhibit bacterial growth. Interpretation of results from this assay was relied on model-dependent analysis, which based on the assumption that antibiotics was diffused freely in the solid nutrient medium. A theoretical description of the agar diffusion assay that was took into consideration the loss of antibiotics during diffusion and provide higher accuracy of the MIC determined from the assay.

RESULTS

Isolation of Bacterial Species

From collected urine sample 98 bacterial strains were streaked in blood, chocolate, MacConkey and cystene lactose electrolyte deficient (CLED) agar for isolation of MRSA, *E. faecalis* and *K. pneumonae.*

Cultured Colonies of Staphylococcus aureus

For *S. aureus*, yellow to cream colony of diameter of 1-2mm was obtained in which pigment was less pronounced in young colonies. Some gave beta-hemolysis in aerobic conditions on blood show in fig 4.1, and on mannitol salt agar (MSA) its showed fermented changed color of MSA red to yellow showed in fig 4.2.

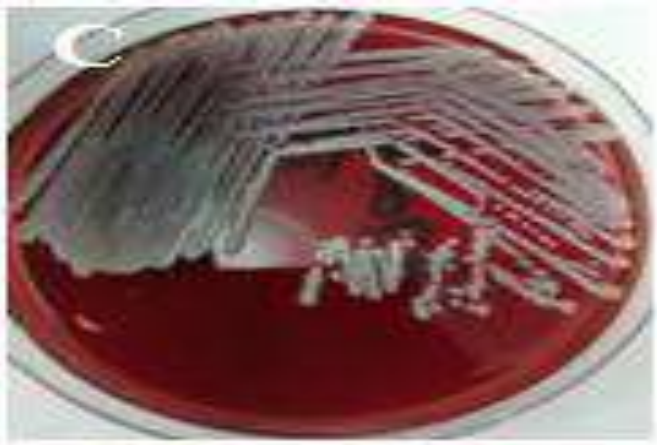


Figure 4.1: Non- hemolytic large grey-white, mucoid colonies on sheep blood agar

Figure 4.2: Non- hemolytic large yellow colonies on MSA

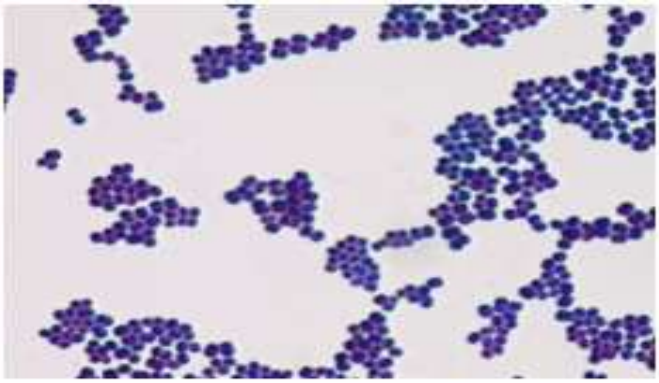


Figure 4.3: Slide of Gram positive bacteria

Biochemical Characterization of Bacterial Species

Colonies of different bacterial and fungal strains obtained on agar plates were then subjected to biochemical characterization as shown in Fig 4.3

Identification Test MRSA

For MRSA confirmation, it was added to DNAs agar media which completely hydrolyzed by the DNases enzyme present in MRSA as shown in the Fig 4.4.

Figure 4.4: Confirmation of MRSA by DNAs Test

Coagulase Test

Coagulase test was performed to identify *S. aureus* in clinical laboratories although had some limitations. About 15% of ordinary strains of MRSA gave negative reactions. So, all coagulase-negative slide was confirmed using a tube coagulase test as the definitive test for *S. aureus* (MRSA) as shown in Fig 4.5.

Figure 4.5*:* Coagulase test positive MRSA

Coagulase test was discriminated pathogenic from nonpathogenic members of the genus *Staphylococcus*. All pathogenic strains of *S. aureus* gave coagulase positive whereas the nonpathogenic species *(S. epidermidis*) gave coagulase negative.

Test for Enterococcus faecalis

E. faecalis was isolated on blood agar and confirmed by bile esculin tests, by showing black colonies.

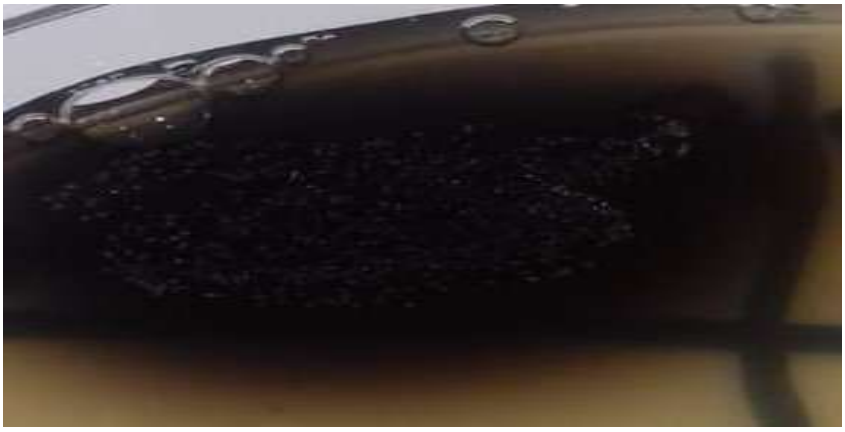


Figure 4.6*:* Black colonies of *E. faecalis*

Diffuse blackening of more than half of the slant within 24-48 hours indicated the esculin hydrolysis. On plates, black haloe was observed around isolated colonies and any blackening was considered positive. Notably, *E. faecalis* hydrolyzed esculin in the presence of bile (4% bile salts or Colonies of *E. faecalis* strain was cultivated on purple agar at 37°C for 24 hrs, color changed around the colonies showed that lactose was fermented during acid production. The total length of the scale bar is equivalent to 10 mm as shown in Fig 4.6.

Identification of Klebsiella pneumonia

Gram negative bacteria were inoculated on MacCkonkey agar to differentiate the Gram –ve strain from Gram +ve strains, *Klebsiella pneumonia* was differentiated because of its morphology its look mucoid and fermented on MacCkonkey agar.

Figure 4.7: Mucoid Klebsiella pneumonae colonies (lactose fermented)

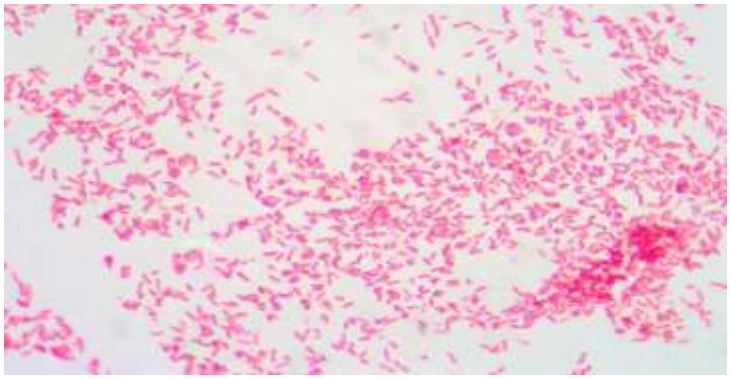


Figure 4.8*:* Slide of Gram negative bacteria

Figure 4.9: The procedure of String Method (Isolation of *Klebsiella*)

The identification of *K. pneumonae* did through API 20 E kit, shown in Figure 4.10. While the test results are given in Table 4.1.

Figure 4.10: Identification of *K. pneumonae* through API 20 E

Biochemical Test Results of K. pneumonae

Table 4.1: Biochemical test results of *K. pneumonae*

Tests	Results	Tests	Results	Tests	Results	Tests	Results
ONPG	-ve	**ADH**	-ve	**LDC**	+ve	**ODC**	-ve
CIT	+ve	**H_2S**	-ve	**URE**	-ve	**TDA**	-ve
IND	-ve	**VIP**	+ve	**GEL**	-ve	**GLU**	+ve
MAN	+ve	**INO**	+ve	**SOR**	+ve	**RHA**	+ve
SAC	+ve	**MEL**	+ve	**AMY**	+ve	**ARA**	+ve

Antibacterial Assessments of Synthetic Antibiotics

Figure 4.11 showed the resistance of cefoxitin antibiotics against MRSA. cefoxitin found resistance against MRSA in UTI samples. Zone size of 8mm, did not fall the criteria of sensitive drug.

Figure 4.11: Cefoxitin placed for MRSA colony

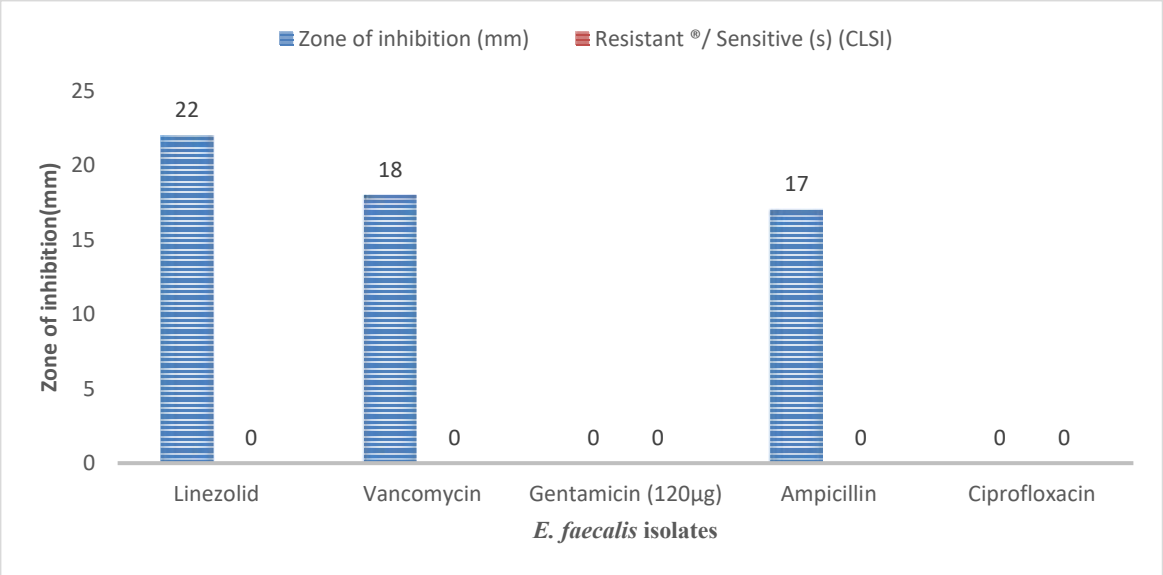
High-level Aminoglycoside Resistant Enterococcus faecalis

E. faecalis was lawend on blood agar plate and different synthetic antibiotics were applied on it by disc diffusion method, *E. faecalis* was resistant against GN (120µG) which is shown in Fig 4.12

Figure 4.12: The resistivity of *E. faecalis* seen against aminoglycoside

Table 4.2: List of Antibiotics actions against *E. faecalis.*

Antibiotics	Zone of inhibition (mm)	Resistant ®/ Sensitive (s) (CLSI)
Linezolid	22	S
Vancomycin	18	S
Gentamicin (120µg)	0	R
Ampicillin	17	S
Ciprofloxacin	0	R



Graph 4.1: Zone size of antibiotics against *E. faecalis*

Extended Spectrum β-Lactamase Resistant K. pneumoniae

In Figure ampicillin, cefepime, ceftazidime and cefotaxime was individually showed their activity against *K. pneumoniae* by synergism. That's why on this basis the bacteria indicated called ESBL.

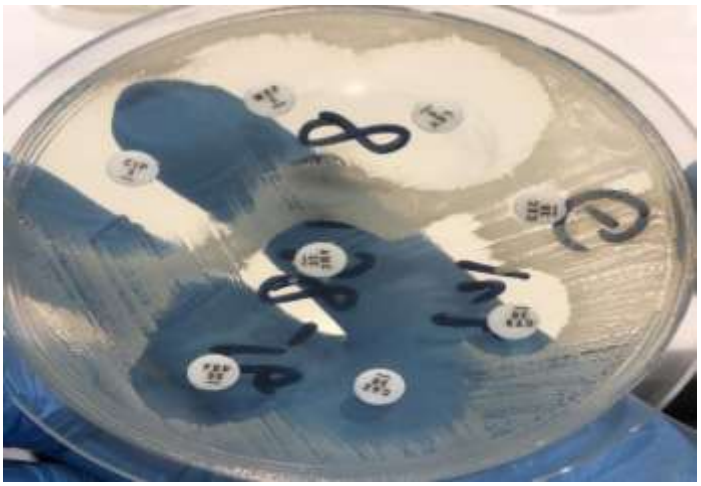


Figure 4.13*:* Antibiotic activity against *K. pneumoniae.*

Antibacterial Activity of Vitamins (B1, B2 and B6)

Vitamins against MRSA

These strain showed resistivity against methicillin and related penicillin, Vancomycin often needed to treat MRSA infection**.**

Figure 4.14: Vitamins (B1, B2, and B6) showed no activity against MRSA.

Vitamins against HLAR E. faecalis

E. faecalis suspension lawend on blood agar, vitamin B1, B2 and B6 were applied on it by well method, they were showed no any synergistic effect.

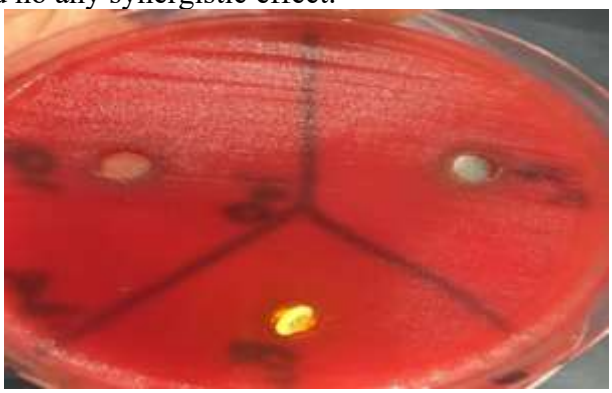


Figure 4.15: Vitamins (B1, B2 and B6) showed no activity against *E. faecalis*

Vitamins against K. pneumoniae

These strain showed resistivity against vitamins B1, B2 and B6

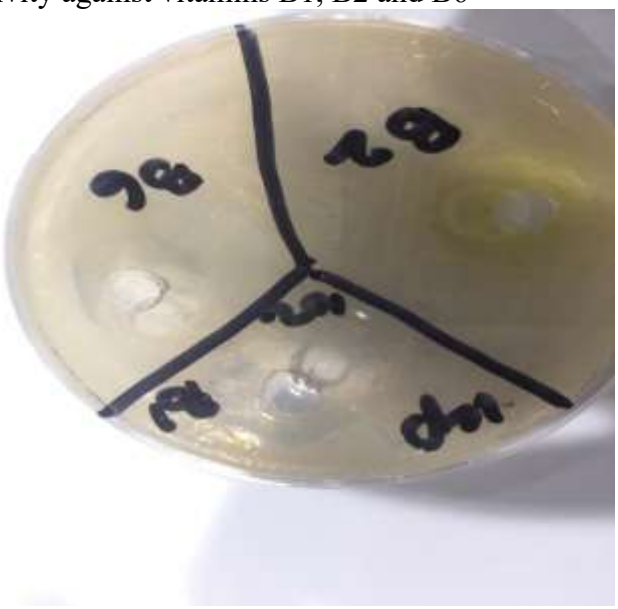


Figure 4.16: Vitamins (B1, B2 and B6) showed no activity against *K. pneumoniae*

Synergistic Effect of Vitamins with Synthetic Antibiotics

MRSA against vitamin and drug

Cefoxitin and B6 in synergism showed antibacterial activity against MRSA.

Figure 4.17: The action of Cefoxitin with B6 against MRSA

Table 4.3: The action of Cefoxitin with B6 against MRSA.

Antibiotic name	Zone size	Action
Cefoxitin	24	Sensitive

HLAR E. faecalis against vitamins and drug

No Synergism of vitamins and gentamicin 120 µg showed antibacterial activity against *E. faecalis*.

Figure 4.18: No synergistic effect of GN120 with vitamin B1, B2 and B6

K. pneumoniae against vitamins and drugs

Zone of cefepime and amoxcilline antibiotics, antibiotics with vitamin B1, B2 and B6 and only vitamins showed no synergistic effect on *K. pneumoniae* strain shown in Figs bellow

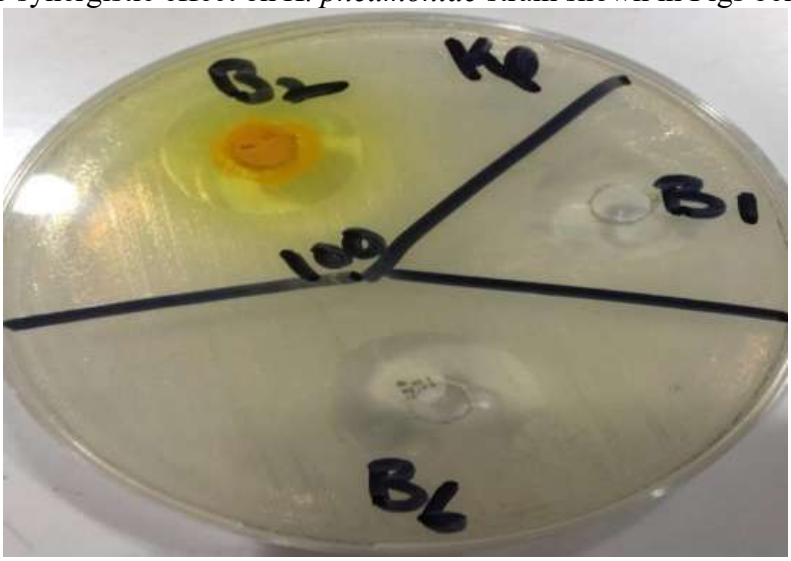


Figure 4.19: Reaction of cefepime with vitamin B1, B2 and B6 show no synergistic effect

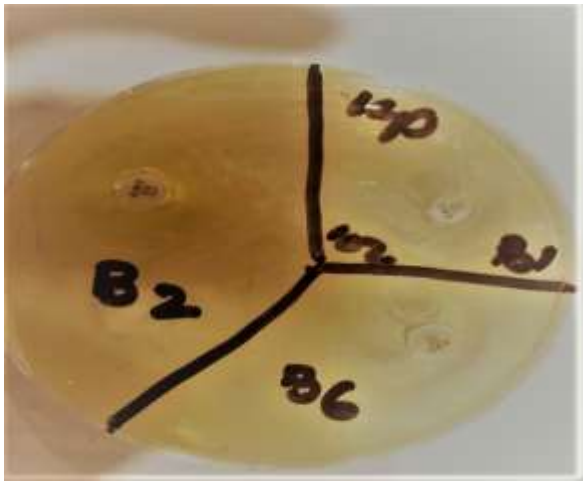
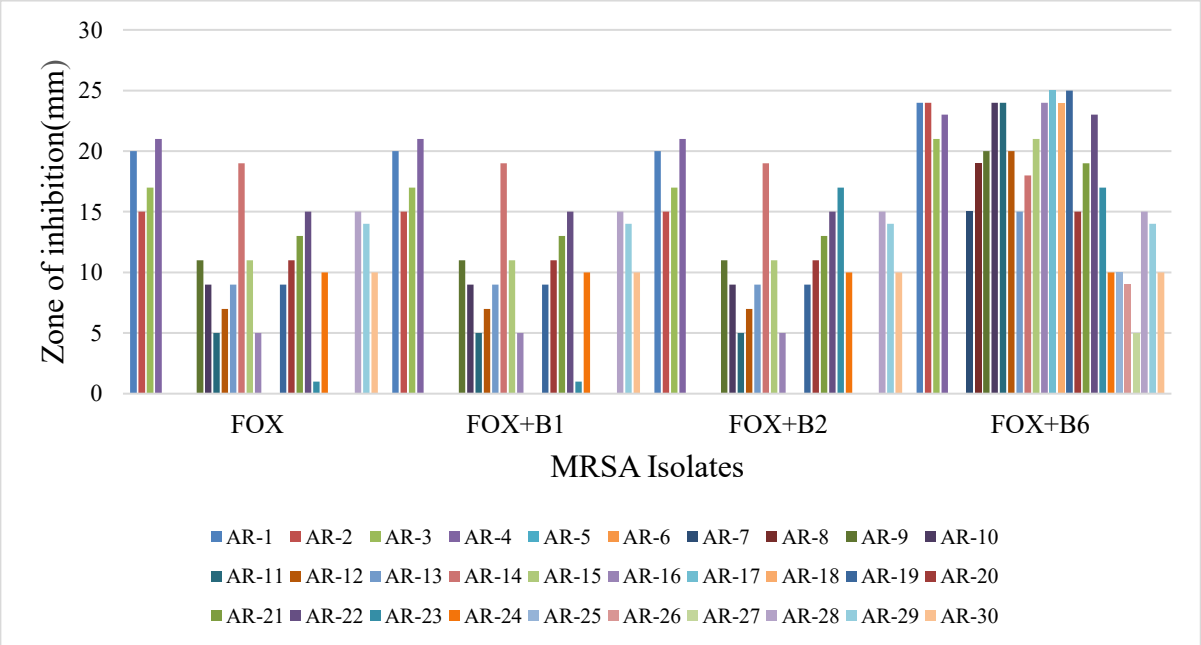


Figure 4.20 Reaction of AMC with vitamin B1, B2 and B6 show no synergistic effect

Table 4.4: MRSA reaction zone size (mm) with Drug and Vitamins

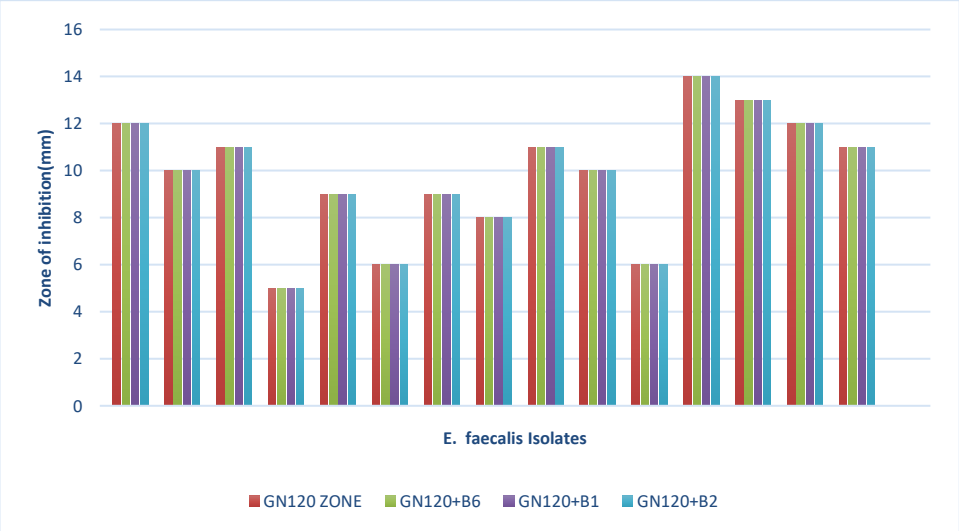
MRSA ISOLATES	FOX	FOX+B1	FOX+B2	FOX+B6
AR-1	20	20	20	24
AR-2	15	15	15	24
AR-3	17	17	17	21
AR-4	21	21	21	23
AR-5	0	0	0	0
AR-6	0	0	0	0
AR-7	0	0	0	15
AR-8	0	0	0	19
AR-9	11	11	11	20
AR-10	9	9	9	24
AR-11	5	5	5	24
AR-12	7	7	7	20
AR-13	9	9	9	15
AR-14	19	19	19	18
AR-15	11	11	11	21
AR-16	5	5	5	24
AR-17	0	0	0	25
AR-18	0	0	0	24
AR-19	9	9	9	25
AR-20	11	11	11	15
AR-21	13	13	13	19
AR-22	15	15	15	23
AR-23	1	1	17	17
AR-24	10	10	10	10
AR-25	0	0	0	10
AR-26	0	0	0	9
AR-27	0	0	0	5
AR-28	15	15	15	15
AR-29	14	14	14	14
AR-30	10	10	10	10



Graph 4.2: Zone size of drugs (mm) bacterial isolates and vitamins with MRSA

Table 4.5: *E. faecalis* reaction zone size (mm) with Drug and Vitamins

E. faecalis Isolates	GN120 ZONE	GN120+B6	GN120+B1	GN120+B2
AR-1	0	0	0	0
AR-2	0	0	0	0
AR-3	0	0	0	0
AR-4	0	0	0	0
AR-5	0	0	0	0
AR-6	0	0	0	0
AR-7	12	12	12	12
AR-8	10	10	10	10
AR-9	11	11	11	11
AR-10	5	5	5	5
AR-11	9	9	9	9
AR-12	6	6	6	6
AR-13	9	9	9	9
AR-14	8	8	8	8
AR-15	11	11	11	11
AR16	10	10	10	10
AR-17	6	6	6	6
AR-18	14	14	14	14
AR-19	13	13	13	13
AR-20	12	12	12	12
AR-21	11	11	11	11
AR-22	0	0	0	0

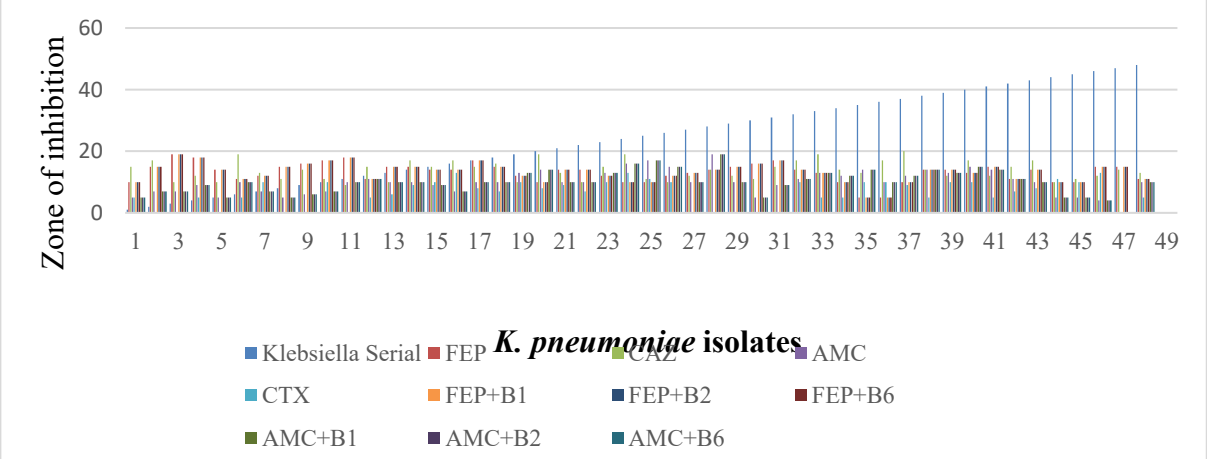


Graph 4.3*: Zone size of drugs and vitamins with *E. faecalis.*

Table 4.6: *K. pneumoniae* reaction zone size (mm) with drug and vitamins

K. pneumonia Isolates	FEP	CAZ	AMC	CTX	FEP+B1	FEP+B2	FEP+B6	AMC+B1	AMC+B2	AMC+B6
AR-1	10	15	5	5	10	10	10	5	5	5
AR-2	15	17	7	0	15	15	15	7	7	7
AR-3	19	10	7	0	19	19	19	7	7	7
AR-4	18	12	9	5	18	18	18	9	9	9
AR-5	14	10	5	0	14	14	14	5	5	5
AR-6	11	19	10	5	11	11	11	10	10	10
AR-7	12	13	7	10	12	12	12	7	7	7
AR-8	15	11	5	0	15	15	15	5	5	5
AR-9	16	14	6	0	16	16	16	6	6	6

AR-10	17	11	7	10	17	17	17	7	7	7
AR-11	18	9	10	0	18	18	18	10	10	10
AR-12	11	15	11	5	11	11	11	11	11	11
AR-13	15	10	10	6	15	15	15	10	10	10
AR-14	15	17	10	9	15	15	15	10	10	10
AR-15	14	15	9	10	14	14	14	9	9	9
AR-16	14	17	7	13	14	14	14	7	7	7
AR-17	17	15	10	8	17	17	17	10	10	10
AR-18	15	16	10	7	15	15	15	10	10	10
AR-19	12	10	13	10	12	12	12	13	13	13
AR-20	10	19	14	8	10	10	10	14	14	14
AR-21	14	13	10	9	14	14	14	10	10	10
AR-22	14	10	10	7	14	14	14	10	10	10
AR-23	12	15	13	10	12	12	12	13	13	13
AR-24	10	19	16	13	10	10	10	16	16	16
AR-25	10	11	17	11	10	10	10	17	17	17
AR-26	12	10	15	10	12	12	12	15	15	15
AR-27	13	12	10	0	13	13	13	10	10	10
AR-28	14	14	19	0	14	14	14	19	19	19
AR-29	15	12	10	0	15	15	15	10	10	10
AR-30	16	11	5	0	16	16	16	5	5	5
AR-31	17	15	9	0	17	17	17	9	9	9
AR-32	14	17	11	10	14	14	14	11	11	11
AR-33	13	19	13	5	13	13	13	13	13	13
AR-34	10	14	12	5	10	10	10	12	12	12
AR-35	5	13	14	10	5	5	5	14	14	14
AR-36	5	17	10	10	5	5	5	10	10	10
AR-37	10	20	12	9	10	10	10	12	12	12
AR-38	14	14	14	5	14	14	14	14	14	14
AR-39	14	12	13	10	14	14	14	13	13	13
AR-40	13	17	15	10	13	13	13	15	15	15
AR-41	15	12	14	5	15	15	15	14	14	14
AR-42	11	15	11	7	11	11	11	11	11	11
AR-43	14	17	10	8	14	14	14	10	10	10
AR-44	10	10	5	11	10	10	10	5	5	5
AR-45	10	11	5	10	10	10	10	5	5	5
AR-46	15	12	4	13	15	15	15	4	4	4
AR-47	15	14	0	0	15	15	15	0	0	0
AR-48	11	13	10	5	11	11	11	10	10	10



Graph 4.4: Zone size of drugs and vitamins with *K. pneumoniae*

Table 4.7. Summary of synergistic effect of drugs and vitamins

Pathogens	MRSA	*Streptococcus*	*Klebsiella* (ESBL+ Persistent Drugs)
No. of Samples	30	22	48
Drug Used	Cefoxitin (FOX)	Gentamycin (GN120)	Amoxicillin (AMC), Cefotaxime (CTX), Ceftazidime (CAZ), Cefepime (FEP)
Vitamins Used with Drugs	FOX + B1, FOX + B2 ,FOX + B6	GN120+B1, GN120+B2, GN120+B6	AMC+B1, FEP+B1, AMC+B2, FEP+B2, AMC+B6, FEP+B6
Results	FOX + B1= Negative FOX + B2=Negative FOX + B6=Positive	No synergistic effect	No synergistic effect
Remarks	FOX+B6 show synergistic effect while others do not show a synergistic effect	No synergistic effect was seen	No synergistic effect was seen

Total 100 sample were analyzed of urinary tract infection, 30 were indicated as positive for MRSA, 22 were diagnosed positive for *E. faecalis* and 48 showed positivity for *K. pneumoniae,* FOX drug was used for MRSA which found resistant. Then sensitivity checked with mixture of FOX+B1, FOX+B2 and FOX+B6 against MRSA. FOX+B6 showed their synergistic effect while others did not show synergism. Their zone size is mentioned in table 07. GN120 drug used for *E. faecalis* which found resistant. Then sensitivity was checked with mixture of GN120+B1, GN120+B2 and GN120+B6 no synergistic effect was seen. Their zone size showed in table 08. AMC, FEP CAZ and CTX drugs used for *K. pneumonia*, these drugs were found to made a ESBL. Then separately sensitivity of AMC and FEP checked with mixture of B1, B2 and B6 against *K. pneumoniae* no synergism was seen. Their zone size is mentioned in Table 4.4, 4.5 and 4.6. A comprehensive picture showed in Table 4.7.

DISCUSSION

Urinary tract infections (UTIs) represent the most common bacterial infections worldwide, imposing a significant financial burden on healthcare systems (Stamm & Norrby, 2001). The challenge in managing UTIs stems from the diversity of uropathogens harboring various virulence factors, compounded by the alarming rise in antimicrobial resistance, which limits the efficacy of antibiotic therapies (Chen, Ko, & Hsueh, 2013; Jacobsen et al., 2008). Recurrent infections further indicate that antibiotics are not universally effective for all UTI cases, underscoring the urgent need for alternative or adjunctive treatment strategies.

Among uropathogens, *Klebsiella pneumoniae* has been extensively studied globally. Resistance rates reported in multiple studies highlight a worrying upward trend. Nasehi et al. (2010) documented resistance rates of 34.7% and 33.5%, whereas Eftekhar et al. (2012) observed higher rates of 49% and 37.2% (Aminzadeh et al., 2008; Gales, Reis, & Jones, 2001). Notably, resistance to broad-spectrum cephalosporins has increased significantly in recent years. Aztreonam exhibited the highest resistance among non-cephalosporin antibiotics, while amikacin showed the lowest resistance rates, aligning with previous reports (Feizabadi et al., 2006; Nathisuwan, Burgess, & Lewis, 2001). Specifically, among third-generation cephalosporins, cefotaxime showed the highest resistance (50%), whereas ceftizoxime demonstrated the lowest (40.2%), possibly due to its less frequent clinical use.

Extended-spectrum β-lactamase (ESBL) production, a major resistance mechanism in *K. pneumoniae*, varies regionally. In this study, 26.3% of isolates exhibited ESBL phenotypes, a rate considerably lower than that reported in India (97.1%), Turkey (57%), and South Korea (30%) (Mansury et al., 2016). Using combined disk and E-test methods, 100% of ESBL producers were resistant to ceftazidime. Other β-lactamase phenotypes showed 81.5-92.1% positivity. Iranian studies report varied ESBL rates: Feizabadi et al. (2006) found 44.5%, Aminzadeh et al. (2008) 52.5%, and Ramazanzadeh et al. reported 34.8% (Aminzadeh et al., 2008). Bazzaz et al. (2009) noted 59.2%

prevalence in *E. coli* and *K. pneumoniae*. This study's isolates resistant to aztreonam further support its role as a sensitive marker for ESBL detection.

Comparing phenotypic confirmatory tests for ESBL detection, ESBL E-test strips slightly increased detection to 38 positive isolates from 35 identified by combined disk methods, consistent with Mohanty et al. (2009). PCR analysis revealed 19.4% of isolates carried the CTX-M gene, 22.2% the SHV gene, and 16% the TEM gene; no PER gene was detected. These findings mirror previous studies in Iran and elsewhere, where SHV predominates, followed by CTX-M and TEM genes (Nasehi et al., 2010; Walsh, 2003). Globally, CTX-M variants are increasingly replacing SHV and TEM as the dominant ESBL genes in Gram-negative bacteria (Bush, 2008).

Regarding methicillin-resistant *Staphylococcus aureus* (MRSA), literature suggests that treatment of MRSA bacteremia is best managed with anti-staphylococcal penicillins or first-generation cephalosporins rather than vancomycin, due to poorer outcomes associated with vancomycin therapy (Chang et al., 2003; Kim et al., 2008; Schweizer et al., 2011). Given MRSA's increasing prevalence in both community and healthcare settings, empirical therapy for Gram-positive bacteremia often includes anti-MRSA agents combined with cephalosporins to enhance antibacterial efficacy (McConeghy, Bleasdale, & Rodvold, 2013). Tang et al. (2017) demonstrated enhanced antibacterial activity of cephalosporin-glycopeptide combinations against clinical MRSA isolates regardless of cephalosporin MIC values.

Vancomycin minimum inhibitory concentrations (MICs) correlate with clinical outcomes in MRSA infections; higher MICs (≥1 μg/mL) are linked to increased mortality and treatment failure (Jacob & DiazGranados, 2013; Haque et al., 2010). Rahman et al. (2004) reported vancomycin efficacy of 55.6% against isolates with MIC < 0.5 μg/mL, dropping to 9.5% at MIC 1-2 μg/mL, highlighting the need for alternative treatments in such cases.

Enterococcus faecalis remains a formidable antibiotic-resistant pathogen due to its rapid acquisition and dissemination of resistance genes, facilitated by pheromone signaling within and across bacterial species (Vickerman et al., 2010). Strains such as *E. faecalis* ATCC 29212 and OG1RF are commonly employed as model organisms for survival and biofilm studies (Bourgogne et al., 2008; Kim et al., 2012). Vancomycin and linezolid, often considered last-resort treatments for resistant Gram-positive infections, demonstrated limited in vitro efficacy against tested *E. faecalis* strains in this study, while penicillin and ampicillin exhibited better antibacterial activity with lower MIC and minimum bactericidal concentrations (MBC) (Weinstein, 2001).

Given the challenges posed by resistant pathogens, research into adjunct therapies such as vitamins has gained traction. Vitamins, especially antioxidants like vitamin C, are known to modulate the immune system and may influence bacterial growth (Ashraf & Sajid, 2018). This study supports earlier findings on the antibacterial roles of vitamins; vitamin A (retinol) exhibited inhibitory effects on bacterial growth, consistent with reports highlighting the antimicrobial activity of retin-aldehyde derivatives (Flemetakis & Tsambaos, 1989; Liggins et al., 2019). Conversely, although vitamin D's in vivo immunomodulatory effects against pathogens like *Mycobacterium* species are well documented (Gombart, 2016; Martineau et al., 2007), this study did not find synergistic in vitro effects of vitamin D against *Acinetobacter baumannii*, likely due to differences in bacterial cell wall structures.

Vitamin E demonstrated significant synergistic effects with multiple antibiotics (imipenem, meropenem, polymyxin B, piperacillin-tazobactam, and amikacin) against *A. baumannii*, which was otherwise resistant to these drugs. This aligns with previous studies showing vitamin E's inhibitory action against *E. coli*, *Pseudomonas*, and *S. aureus* (Valentin & Qi, 2005; Tintino et al., 2016). Vitamin E's antioxidant properties may contribute to its antimicrobial effects via immune modulation.

Vitamin K also showed remarkable synergy with several antibiotics against *A. baumannii*, likely by increasing bacterial membrane permeability due to its lipophilic nature (James, Weaver, & Cantorna, 2017). However, vitamin K exhibited negligible synergism against MRSA, highlighting the differential effects of vitamins based on bacterial cell wall composition.

Among water-soluble vitamins, this study found notable synergistic activity of vitamins B1, B2, B6, and B12 with various antibiotics. Vitamin B2 (riboflavin) showed broad synergism against MRSA, consistent with its known antimicrobial role, especially when photoactivated (James et al., 2017). Vitamin C synergized with multiple antibiotics, corroborating prior research that demonstrated its bacterial growth inhibition properties (Taha et al., 2019). The combination of vitamin B6 with cefoxitin notably enhanced activity against MRSA, suggesting potential clinical relevance for managing resistant infections.

In contrast, ESBL-producing *K. pneumoniae* isolates did not show susceptibility when antibiotics were combined with vitamins B1, B2, or B6. Similarly, high-level aminoglycoside-resistant *E. faecalis* showed no improvement with gentamicin combined with these vitamins, indicating limitations to vitamin-antibiotic synergy depending on resistance mechanisms.

Collectively, these findings emphasize the potential of integrating vitamins with synthetic antibiotics as a novel therapeutic approach to combat multidrug-resistant bacterial infections. Given the escalating resistance rates and limited new antibiotic development, such strategies could be particularly valuable in resource-limited settings like Pakistan, offering cost-effective adjunct treatments. Future research should explore the molecular mechanisms underlying vitamin-antibiotic interactions and assess their efficacy at the genetic level to facilitate development of more potent, broad-spectrum antimicrobial agents.

CONCLUSION

In current study, antibiotics with vitamins showed synergistic activity. Vitamin B6 with cefoxitin showed maximum activity against MRSA. So it was concluded that the efficacy of synergism of synthetic antibiotics and vitamins was assessed as a promising antibacterial approach for the identified resistant bacteria strain.

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