International Journal of Pathogen Research

7(1): 37-46, 2021; Article no.IJPR.68900
ISSN: 2582-3876

In Vitro Effects of Vitamin and Mineral Supplements on Antibiotic Resistance Profile of Some ESKAPE Pathogens

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Authors' contributions

This work was carried out in collaboration among all authors. Authors OCE and EAE designed the study. Author OCE performed the statistical analysis, wrote the protocol, managed the literature searches and wrote the first draft of the manuscript. Authors DCE and EAE managed the analyses of the study and edited the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPR/2021/v7i130175
Editor(s):
(1) Prof. John Yahya I. Elshimali, UCLA School of Medicine and Charles R. Drew University of Medicine and Science, California, USA.
Reviewers:
(1) Hussein O. M. Al-Dahmoshi, University of Babylon, Iraq.
(2) Aneela Khawaja, Rahbar Medical and Dental College, Pakistan.
Complete Peer review History: http://www.sdiarticle4.com/review-history/68900

Original Research Article

Received 01 April 2021
Accepted 06 June 2021
Published 11 June 2021

ABSTRACT

Background: Antibiotics once seen as miracle drugs are now becoming inefficient in treating various bacterial diseases. This study aimed to evaluate the effects of vitamin and mineral supplements on the antibiogram profile of some of the multidrug-resistant bacteria, which the Infectious Diseases Society of America (IDSA) has dubbed ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter* spp*, Pseudomonas aeruginosa, and Enterobacter* spp), the predominant cause of hospital-acquired infections (HAIs).
Methodology: The *in vitro* effects were evaluated using the disc diffusion (Kirby-Bauer) technique. All test bacteria were inoculated onto Mueller-Hinton agar (MHA), supplemented with varying concentrations (2.5, 5, 10, 20, and 25 mg/ml) of vitamin (A, C, or E) and mineral (calcium or iron). Agar without supplements served as the control. The effects of vitamin and mineral supplements were determined by measuring the zones of inhibition to the nearest millimeter as compared to the control.

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Result: Zones of inhibition for nalidixic acid and ampicillin on *P. aeruginosa* significantly increased from 5mm to 32mm and 0mm to 18mm respectively, with increasing concentration of vitamin C. Similarly, nalidixic acid and ampicillin zones of inhibition on *P. aeruginosa* increased from 5mm to 12mm and 0mm to 18mm respectively, with increasing concentration of vitamin A. Vitamin C resulted in significant decreases in all of the zones of inhibition for all antibiotics against *E. coli,* except reflacine and ciproflox. Varying concentrations of iron led to a sharp decrease in the zones of inhibition for all antibiotics against *S. aureus and K. pneumonia*. Significant changes were also observed in all zones of inhibitions for all antibiotics studied under varying concentrations of calcium.

Conclusion: The effects of vitamin and mineral supplements appear to be important but concentration-dependent. However, there is a need to evaluate the *in vivo* effects of these vitamin and mineral supplements.

Keywords: Antibiotic resistance; ESKAPE pathogens; vitamin and mineral supplements.

1. INTRODUCTION

The Infectious Diseases Society of America (IDSA) highlighted a clique of microorganisms – acronymically termed 'the ESKAPE pathogens' – capable of 'escaping' the biocidal action of antibiotics and jointly representing new patterns in pathogenesis, transmission, and resistance [1]. ESKAPE represents a group of bacteria, encompassing both Gram-positive and Gram-negative species, made up of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp [1,2]. These ESKAPE pathogens are the predominant cause of life-threatening nosocomial infections amongst critically ill and immune-compromised individuals and are characterized by potential drug resistance mechanisms [1,2]. It is not only enormously difficult to treat infections caused by multidrug-resistant pathogens, but also these infections can spread throughout a hospital or community [3]. The immediate introduction of novel compounds is widely accepted as the conventional response to temporarily ease the concerns of modern medicine for emerging resistance [4]. It has been prognosticated that the lack in the availability of such compounds would have myriad consequences on a global scale, not only on public health but furthermore [5]. Also, some novel antibiotics have faced major setbacks and are expensive [6].

Investment in novel antimicrobial research and development (R&D) by pharmaceutical companies has faced major setbacks in the last two decades. Consequently, this has resulted in a considerable decline in novel antibiotics, and a production rate failing to keep up with the resulting increase in antibiotic resistance around the globe [7,8]. Having acknowledged that an empty 'pipeline' leads to devastating future of a 'post-antibiotic era' [8], key health authorities including the WHO, the European Centre for Disease Prevention and Control, and the Infectious Diseases Society of America (IDSA) have encouraged industrial incentives and initiatives in view to promote research into novel antimicrobial compounds and safeguard the remaining therapeutic agents available to physicians and encourage the efforts against key human pathogens [9].

Data from reported clinical trials and laboratory studies suggest that some vitamin and mineral supplements play a crucial role in health [10-13] and are sometimes used in combination with antibiotics in the treatment of infections [14]. Furthermore, the study of Majed et al. suggests that the consumption of vitamins or any other supplements with antibiotics sometimes decreases the antibacterial activity of many antibiotics [14]. These findings gave incentive to this study, which was designed to find alternative clinical applications that may help in the treatment of infections caused by multidrug-resistant pathogens. In this study, we demonstrated the *in vitro* effects of increasing concentrations of vitamin and mineral supplements on some ESKAPE pathogens responsible for nosocomial infections.

2. MATERIALS AND METHODS

2.1 Study Period and Duration

The study duration was April–August 2019 during which we collected data, analyzed results, and did the statistical analysis. In April 2019, we started procuring kits, supplements and reagents, and our test organisms.

2.2 The Test Organisms

The test bacterial organisms were pure clinical isolates obtained from the Diagnostic Microbiology Laboratory, Departments of Microbiology, University of Nigeria, Nsukka. These include one isolate each of *Staphylococcus aureus, Klebsiella pneumoniae*, *Pseudomonas aeruginosa, and Escherichia coli.* All clinical isolates were stored at 4°C on nutrient agar slants. Subculture was done every 30 days to maintain viability and purity.

2.3 Vitamin and Mineral Supplements

Vitamin A, C, and E were the vitamin supplements used for this study. While Calcium and Iron served as mineral supplements. Mueller-Hinton agar (MHA) was supplemented with varying concentrations (2.5mg/ml, 5mg/ml, 10mg/ml, 20mg/ml and 25mg/ml) of the selected vitamin and mineral.

2.4 Evaluation of the Effects of Vitamin and Mineral Supplements

The effects of vitamin and mineral supplements on the ESKAPE pathogens were determined using the disk diffusion method. The procedure was carried out following the guidelines provided by the Clinical Laboratory Standard Institute [15]. Five (5) sets of freshly prepared 18ml Mueller-Hinton agar (MHA) were supplemented separately with one of the vitamins or mineral supplements such that the final concentrations were 2.5mg/ml, 5mg/ml, 10mg/ml, 20mg/ml and 25mg/ml respectively. Equally, a set of 18ml MHA without supplements served as controls. The plates containing the various concentrations of the supplement were inoculated with an 18h old standardized inoculum (10^8 CFU/ml) of the test bacteria by streaking with a sterile cotton-tipped swab to achieve confluent growth. The inoculated plates were allowed to dry after which the antibiotic discs were placed on the surface of the agar using sterilized forceps. Subsequently, the plates were carefully inverted and then incubated at 37^oC overnight, under aerobic conditions. After incubation, the diameter of the zones of inhibition was measured to the nearest millimeter and recorded.

2.5 Statistical Analysis

All data were analyzed for statistical significance difference using one–way analysis of variance of a Statistical Product and Service Solutions (SPSS) software version 20.0 (SPSS, 2011). $p < 0.05$ was considered statistically significant.

3. RESULTS

The effects of vitamin C supplements on ESKAPE pathogens are presented in Table 1. The zones of inhibition for all antibiotics except streptomycin and rifampicin against *S. aureus* showed a progressive decrease, with increasing concentration of vitamin C. Our study observed a decrease in all zones of inhibitions for all antibiotics except nalidixic acid and ampicillin. Importantly, nalidixic acid and ampicillin zones of inhibition significantly increased from 5mm to 32mm and 0mm to 18mm respectively ($p < 0.05$), with increasing concentration of vitamin C on *P. aeruginosa*. All zones of inhibition were affected by the different concentrations of vitamin C on *K. pneumonia*. With increasing concentration of vitamin C on *E. coli,* we observed significant decreases ($p < 0.05$), in all of the zones of inhibition for all antibiotics except reflacine and ciproflox.

Table 2 shows the effects of vitamin A on ESKAPE pathogens. A decrease in the zones of inhibition for all antibiotics against *S. aureus* except rifampicin was observed ($p < 0.05$). All zones of inhibitions for all antibiotics showed a progressive fall with increasing concentration of vitamin A against *P. aeruginosa*, except nalidixic acid and ampicillin. Nalidixic acid and ampicillin zones of inhibition increased from 5mm to 12mm and 0mm to 18mm respectively ($p < 0.05$), with increasing concentration of vitamin A on *P. aeruginosa*. The study observed notable effects of vitamin A supplement on the resistance pattern of *K. pneumoniae*, the zone of inhibition of ciproflox showed a large increase. Varying concentrations of vitamin A affected the zones of inhibitions of all antibiotics on *E. coli* as shown in Table 2.

Table 3 presents the effects of vitamin E on ESKAPE pathogens. Vitamin E supplement resulted in an increase in the zones of inhibition for the majority of the antibiotics against *S. aureus.* We also observed changes in the zones of inhibition of all antibiotics with varying concentrations of vitamin E on *P. aeruginosa, K. pneumonia, and E. coli* (Table 3). The effects of iron on ESKAPE pathogens in Table 4 show a sharp decrease in the zones of inhibition for all antibiotics against *S. aureus*. In comparison to the control, notable changes were also noted with varying concentrations of the iron

supplement on *P. aeruginosa.* Significantly, the effectiveness of ampicillin against *P. aeruginosa,* with varying concentrations of iron, increased from 0mm to 26mm ($p < 0.05$). Zones of inhibition of all test antibiotics showed a progressive decrease with increasing concentration of iron on *K. pneumonia.* In comparison to the control, all zones of inhibitions for all antibiotics, studied in varying concentrations of calcium, showed significant changes as shown in Table 5 ($p < 0.05$).

4. DISCUSSION

The development of resistance by a pathogen to many of the widely used antibiotics provides an incentive for further attempts to search for novel antimicrobials, and novel approaches to developing them are urgently needed [16]. Similarly, veritable containment strategies are continually being thought of and evaluated. The results of this study showed appreciable and significant effects in the zones of inhibition of all test antibiotics against *S. aureus, P. aeruginosa, K. pneumoniae, and E. coli* in media containing the supplements, as compared to the respective controls. These varying changes in the zones of inhibition of all antibiotics suggest that vitamins A, C, and E and mineral supplements; iron and calcium have *in vitro* effects on the antibiogram profile of some ESKAPE pathogens.

Table 1. Effects of Vitamin C Supplement on ESKAPE Pathogens

	Zone of inhibition (mm)					
Concentration of supplement(mg/ml)	0.00	2.5	5	10	20	25
S. aureus						
Ciproflox	26	26	24	26	0	0
Gentamycin	28	14	10	8	0	0
Amoxil	20	0	0	0	0	0
Streptomycin	28	0	0	12	22	28
Rifampicin	20	22	22	18	20	20
Chloramphenicol	28	24	24	22	0	0
P. aeruginosa						
Reflacine	28	28	32	24	16	13
Augumentin	14	14	10	8	0	0
Ciproflox	32	32	32	32	20	7
Gentamycin	30	26	22	18	10	0
Nalidixic acid	5	0	0	0	32	32
Ampicillin	0	0	0	0	8	18
K. pneumoniae						
Reflacine	24	30	28	26	18	13
Augumentin	12	0	0	0	0	0
Ciproflox	22	21	24	30	24	20
Gentamycin	14	30	26	20	18	18
Nalidixic acid	12	0	0	0	0	0
Ampicillin	10	7	0	0	0	0
E. coli						
Reflacine	30	27	24	24	24	20
Augumentin	14	7	0	0	0	0
Ciproflox	28	24	24	24	24	18
Gentamycin	20	0	0	8	10	18
Nalidixic acid	16	0	0	0	0	0
Ampicillin	24	8	0	0	0	0

Key: 0.00mg/ml is control (i.e. without supplement)

Table 2. Effects of Vitamin A Supplement on ESKAPE Pathogens

	Zone of inhibition (mm)					
Concentration of supplement(mg/ml)	0.00	2.5	5	10	20	25
S. aureus						
Ciproflox	26	32	28	18	0	0
Gentamycin	28	32	30	14	0	0
Streptomycin	28	28	26	16	0	0
Amoxil	20	18	21	24	22	28
Chloramphenicol	28	26	26	14	20	20
Rifampicin	20	18	20	16	0	0
P. aeruginosa						
Reflacine	28	20	16	12	18	18
Augumentin	14	13	8	10	10	7
Ciproflox	32	32	28	26	20	15
Gentamycin	30	14	14	14	10	5
Nalidixic acid	5	6	8	8	8	12
Ampicllin	0	0	0	0	8	18
K. pneumoniae						
Reflacine	24	28	25	16	14	13
Augumentin	12	14	10	22	22	18
Ciproflox	22	27	27	26	28	32
Gentamycin	14	24	22	22	28	8
Nalidixic acid	12	6	0	0	8	8
Ampicillin	10	7	0	0	0	0
E. coli						
Reflacine	30	22	22	12	28	28
Augumentin	14	14	12	13	8	6
Ciproflox	28	32	32	32	28	32
Gentamycin	20	30	30	28	24	20
Nalidixic acid	16	7	12	14	20	24
Ampicillin	24	15	18	22	22	21

Key: 0.00mg/ml is control (i.e. without supplement)

Table 3. Effects of Vitamin E Supplement on ESKAPE Pathogens

	Zone of inhibition (mm)					
Concentration of supplement(mg/ml)	0.00	2.5	5	10	20	25
S. aureus						
Ciproflox	26	26	26	26	28	28
Gentamycin	28	8	12	20	22	22
Streptomycin	28	18	24	26	26	28
Amoxil	20	10	12	20	22	22
Chloramphenicol	28	28	26	26	26	20
Rifampicin	20	20	16	20	20	22
P. aeruginosa						
Reflacine	28	28	18	18	20	13
Augumentin	14	14	14	22	22	26
Ciproflox	32	27	24	28	28	30
Gentamycin	30	24	20	22	28	32
Nalidixic acid	5	6	8	8	8	10
Ampicillin	0	4	8	8	8	13
K. pneumoniae						

Reflacine	24	12	14	14	22	24
Augumentin	12	10	12	26	24	28
Ciproflox	22	8	12	20	22	22
Gentamycin	14	18	24	26	26	28
Nalidixic acid	12	28	26	26	26	20
Ampicillin	10	20	22	30	30	32
E. coli						
Reflacine	30	12	14	14	22	24
Augumentin	14	10	12	26	24	28
Ciproflox	28	8	12	20	22	22
Gentamycin	20	18	24	26	26	28
Nalidixic acid	16	28	26	26	26	20
Ampicillin	24	20	22	30	30	32

Key: 0.00mg/ml is control (i.e. without supplement)

Table 4. Effects of Iron Supplement on ESKAPE Pathogens

	Zone of inhibition (mm)					
Concentration of supplement(mg/ml)	0.00	2.5	5	10	20	25
S. aureus						
Ciproflox	26	26	24	14	12	4
Gentamycin	28	26	18	12	14	12
Streptomycin	28	28	28	24	12	4
Amoxil	20	20	20	12	12	8
Chloramphenicol	28	20	16	12	12	8
Rifampicin	20	20	16	18	18	18
P. aeruginosa						
Reflacine	28	22	22	12	28	28
Augumentin	14	32	30	32	32	32
Ciproflox	32	32	32	32	28	32
Gentamycin	30	30	30	12	24	26
Nalidixic acid	5	7	12	14	20	24
Ampicillin	0	15	18	22	22	26
K. pneumoniae						
Reflacine	24	21	20	28	8	5
Augumentin	12	30	26	24	0	0
Ciproflox	22	22	24	24	14	8
Gentamycin	14	27	24	26	12	0
Nalidixic acid	12	12	12	20	0	0
Ampicillin	10	26	24	10	10	0
E. coli						
Reflacine	30	8	12	24	24	24
Augumentin	14	14	10	0	0	0
Ciproflox	28	26	24	24	24	32
Gentamycin	20	18	14	8	10	10
Nalidixic acid	16	5	10	0	0	0
Ampicillin	24	14	10	0	0	0

Key: 0.00mg/ml is control (i.e. without supplement)

Table 5. Effects of Calcium Supplement on ESKAPE Pathogens

	Zone of inhibition (mm)					
Concentration of supplement(mg/ml)	0.00	2.5	5	10	20	25
S. aureus						
Ciproflox	26	32	30	26	20	18
Gentamycin	28	28	24	18	14	18
Streptomycin	28	26	24	22	28	30
Amoxil	20	24	24	22	20	12
Chloramphenicol	28	28	28	26	14	8
Rifampicin	20	16	16	14	12	12
P. aeruginosa						
Reflacine	28	17	16	18	28	32
Augumentin	14	14	14	26	28	28
Ciproflox	32	15	12	26	16	20
Gentamycin	30	12	12	20	30	32
Nalidixic acid	5	4	0	10	0	0
Ampicillin	0	0	0	14	0	0
K. pneumoniae						
Reflacine	24	24	30	24	30	30
Augumentin	12	11	14	14	18	32
Ciproflox	22	22	22	26	26	27
Gentamycin	14	20	20	28	28	30
Nalidixic acid	12	12	16	30	24	20
Ampicillin	10	10	12	28	18	10
E. coli						
Reflacine	30	30	30	30	30	30
Augumentin	14	30	28	20	16	18
Ciproflox	28	28	28	28	26	23
Gentamycin	20	30	30	28	30	8
Nalidixic acid	16	28	22	20	16	8
Ampicillin	24	18	16	20	18	15

Key: 0.00mg/ml is control (i.e. without supplement)

Results from various proof-of-concept studies have demonstrated similar significant effects of vitamin C supplement on various bacteria species such as; *K. pneumoniae and E. coli* [17], *S. aureus* [18], *Helicobacter pylori and Campylobacter jejuni* [19], *Bacillus cereus* [8,20] and *Mycobacterium tuberculosis* [21] and on fungi such as *Candida albicans*[18], *Aspergillus niger*, and *A. flavus*.[22]. Comparatively, our study showed significant *in vitro* effects of vitamin C on the zones of inhibition of all antibiotics against *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and *E. coli*. The study of Isela et al. demonstrated the inhibitory effect of vitamin C on biofilm formation with bacteria such as *Streptococcus mutans, Porphyromonas gingivalis, E. faecalis,* and *S. aureus* [18], while Li et al. demonstrated a similar effect on the enzymatic activity of *Streptococcus pneumoniae* hyaluronate lyase [23]. On the other hand, the studies of Zhang et al. [19] and Habash et al. [24] failed to observe an effect on *E. coli, P. aeruginosa, S. epidermidis,* and *Candida albicans.* Differences in the experimental methods or differences in the concentrations of the supplement used could be the reason behind these contradicting results. Interestingly, these findings are in tandem with the observations made by some studies, that vitamin supplements show considerable activity against Gram-positive bacteria than Gram-negative bacteria, due to the double-layered membrane barrier of Gram-negative bacteria [8,17].

In our study, we observed that the *in vitro* effects of vitamin A were independent of the antibiotic susceptibility pattern of the ESKAPE organisms. We witnessed a progressive fall in the zones of inhibition for all antibiotics, except rifampicin, with increasing concentration of vitamin A against *S.*

aureus (Table 2). Significantly, there were also progressive increases in the zones of inhibition of nalidixic acid and ampicillin from 5mm to 12mm and 0mm to 18mm respectively, against *P. aeruginosa* with increasing concentration of vitamin A. Similar to the findings of Masadeh et al. [25], there was significant increase in the zones of inhibition of all antibiotics against *S. aureus* and *P. aeruginosa*, except reflacine, with increasing concentration of vitamin E. Also, the supplementation of vitamin E led to an increase in the zones of inhibition of ampicillin: 10mm to 32mm, augmentin: 12mm to 28mm, and gentamycin: 14mm to 28mm against *K. pneumoniae* (Table 3). There were also notable changes in the zones of inhibition of all antibiotics against *E.coli*.

Some studies have offered reasons for the *in vitro* effects of vitamin supplements on the bacterial cell. The effects were linked with the structural changes in bacteria, such as irregularly constricted cells observed by phase-contrast microscopy [19], or elongated cells with disorganized membranes seen under the scanning electron microscope [26]. In a similar vein, Novak and Fratamico suggested that the *in vitro* activities of vitamin C on bacteria may be due to its anti-quorum sensing activity [27]. Additional explanations include the presence of antioxidants, flavonoids, and phenolics in vitamin C [18], or the ability of supplements to lower the pH [26], as reported by Habash et al. [28] that vitamin C intake produced acidic urine in subjects. The lowered pH might be responsible for the enhanced activity of some antibiotics used in this study, particularly; ampicillin, augmentin, gentamycin, and nalidixic acid.

Our investigation also manifested a sharp decrease in the zones of inhibition of all antibiotics against *S. aureus*, *K. pneumoniae,* and *E. coli* with increasing concentration of iron (Table 4). Against *P. aeruginosa* (Table 4), the zones of inhibition of the following antibiotics showed a progressive increase with increasing concentration of iron: nalidixic acid from 5mm to 24mm and ampicillin from 0mm to 24mm. With increasing concentration of calcium supplement, increasing zones of inhibition were observed for all antibiotics except ampicillin against *K. pneumoniae*. Comparatively, the majority of the antibiotics studied against *S. aureus*, *P. aeruginosa*, *E. coli* showed decreasing zones of inhibition with increasing concentration of calcium (Table 5).

5. CONCLUSION

This study has shown that vitamin (A, C, and E) and mineral (calcium and iron) supplements exert concentration-dependent effects on the zones of inhibition of antibiotics on some ESKAPE pathogens, and are notably independent of the antibiogram profile of the organisms. It should be noted that these vitamin and mineral supplements are convenient and relatively inexpensive. Varying concentrations of the supplements had significant effects by either decreasing or increasing the zones of inhibition of the antibiotics on the test organisms. Further works are also needed to study the efficacy of other antibiotics against ESKAPE pathogens and other pathogens of clinical importance, in the presence of vitamin and mineral supplements. Most importantly, there is a need to evaluate the in vivo effects of these vitamin and mineral supplements on ESKAPE pathogens, probably using laboratory animals.

ACKNOWLEDGEMENT

The authors are thankful to the technical staff and Department of Microbiology, University of Nigeria Nsukka for providing the facilities to carry out this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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