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# An invitro study of synergetic effect of Vitamin C on antimicrobial activity of antibiotics against *Klebsiella pneumoniae* isolated from respiratory samples

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ABSTRACT

Vitamins are essential for various biological responses such as immune function and gene expression in humans. The infections of respiratory tract with *Klebsiella pneumoniae* is quite common throughout the world. Vitamin C supplements reduces the duration and symptoms of the respiratory infections shown by few therapeutic trials. This study was undertaken to find out whether Vitamin C has got any effect upon the sensitivity pattern of antibacterial prescribed for *Klebsiella pneumoniae* isolated from respiratory samples using disc diffusion method in vitro. In our study, by using student T test analysis, Vitamin C significantly decreased the inhibition zone of antibiotics Ciprofloxacin (40%), Imipenem (72%) indicating a decrease in their antibacterial effect invitro. Also, Vitamin C enhanced the antibacterial effect of Doxycycline (64%) and Ceftazidime-Clavulanic acid (36%) significantly. Significant decrease in zone diameter was observed on addition of Vitamin C to antibiotics, Imipenem (56%), Ciprofloxacin (40%). It is to be noted that effect of Vitamin C on Ceftazidime-Clavulanic acid is both increase and decrease in zone diameter which were also statistically significant. This might be due to small sample size of isolates (Number – 50) undertaken in the study. The observations noted in this study need further development in terms of increased number of isolates and proper standardization of Vitamin concentrations, with respect to each antibiotic. Being an invitro study, it does not establish any chemical interactions between Vitamins and antibiotics and needs further research by clinical methods.

**Key Messages**: The interaction of Vitamin C on antibacterial against common respiratory pathogens needs further exploration in the area of research as they show interesting results when studied invitro under a small sample size.



## 1. Introduction

Vitamins are essential for various biological responses such as immune function and gene expression in the human body.[1] Vitamin C, a water-soluble vitamin discovered in 1920 by Albert Szent-Györgyi in Hungary.[2] Vitamin C is essential for collagen biosynthesis and repair, wound healing, development of bones, synthesis of carnitine, steroids and catecholamines, metabolism of amino acids, cholesterol and iron absorption.[3] Several studies revealed that vitamin C possesses antimicrobial properties and also immunomodulatory functions.[4] Even though majority of vertebrates can synthesize their own vitamin C from glucose, mammals such as guinea pigs and humans lack this feature due to absence of L-glucono-$\gamma$-lactone oxidase, which is necessary for the synthesis of vitamin C in vivo.[5] Therefore, humans need supplementation of vitamin C in diet of about 100 to 200 mg per day.[6,7] The deficiency of Vitamin C can lead to suppressed immune response,[8] higher susceptibility

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to infections,[9] disruption of other normal biological responses and a devastating disease called Scurvy. The infections of respiratory tract are quite common in adults and children worldwide, with *Klebsiella pneumoniae* being one of the important pathogens. Pneumonia is the most frequent nosocomial infection (30 to 33% of cases)[10] and extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriage of K. pneumoniae and the development of multidrug resistant strains such as extended–spectrum betalactamase (ESBL) producers, AmpC producers and metallobetalactamase (MBL) producers.[11] Vitamin C supplementation is also widely given during respiratory tract infections by various clinicians.[12] Vitamin C supplements reduces the duration and symptoms of the upper respiratory infections in regular supplementation trials and in therapeutic trials.[13] The treatments against infections caused by microbial agents are strongly dependent on the sensitivity report from each laboratory. The sensitivity testing is most commonly done by disc diffusion method with the interpretation of zone size, based upon CLSI guidelines.[14] Being the most commonly prescribed supplements in respiratory tract infections, this study was undertaken to find out whether Vitamin C has got any effect upon the sensitivity pattern of antibacterial prescribed for *Klebsiella pneumoniae* isolated from respiratory samples using disc diffusion method in vitro.

## 2. Materials and Methods

Ethical committee clearance was obtained with waiver of consent, as only samples in laboratory were collected for the study. A prospective study was done for the period of two months with 50 isolates of Klebsiella pneumoniae isolated from respiratory samples were taken for this study. The sputum samples requested for bacterial culture were collected in a sterile container. The sputum samples were processed in Blood agar, Chocolate agar and MacConkey agar at $37^0$C for 24 - 48 hours. *Klebsiella pneumoniae* grown in culture were isolated and identified by gram staining, colony morphology and standard biochemical tests. The antibiotic sensitivity pattern was performed by Kirby – Bauer disc diffusion method on Mueller Hinton agar by incubating them at $37^0$C for 24 hours. The standardized antibiotics discs (HIMEDIA) used in this study were – Ciprofloxacin (5mcg), Doxycycline (30mcg), Gentamicin (10mcg), Imipenem (10mcg), Piperacillin-tazobactam (100/10 mcg), Ceftazidime (30mcg), Ceftazidime – Clavulanic acid (30/10mcg) and Chloramphenicol (30mcg). The sensitivity pattern was recorded as per standard Clinical Laboratory Standards Institute (CLSI) guidelines were taken as standard neat zone readings.[14] Extended spectrum betalactamase (ESBL) production was detected by using Ceftazidime (30mcg) and Ceftazidime – Clavulanic acid (30/10mcg). Commercially available solutions of Vitamin C were obtained, with each ml containing 100mg of Ascorbic acid.

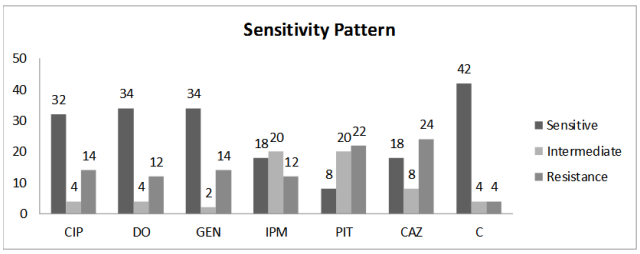
To test the Vitamins, a massive seeding was done from the *Klebsiella pneumoniae* isolates on Mueller Hinton (MH) agar in two plates, using a sterile swab and allowed to dry before placing the discs. The discs with standard antibiotics were then gently placed on agar plates. In the first plate antibiotic sensitivity pattern were performed with antibiotic discs alone for each of the isolate of *Klebsiella pneumoniae*. In the second plate 10$\mu$l (1mg) of Vitamin C solution was pipetted out and added to each of the antibiotic discs and the MH plate was incubated at 35 ± $2^o$C, ambient air for 24 hours. The readings of second group were indicative of combination of Vitamin C and Antibiotics.

### 2.1. Interpretation of Results

The diameter of the inhibition zones was measured in millimeter after 24 hours of incubation using a standard scale. The zone diameter of individual antibiotics in sensitivity pattern testing and the zone diameter of antibiotics in combination with Vitamin C were recorded. Any increase, decrease or no change in zone size of vitamin C and antibiotic combination in comparison with individual antibiotics were noted. The differences in zones were statistically analyzed by student t test for significance.

## 3. Results

The complete sensitivity patterns of isolates are illustrated in Figure 1 below. The antibiotic sensitivity of 50 *K.pneumoniae* showed that, most of the isolates were sensitive to antibiotics, Chloramphenicol (42 isolates), Doxycycline (34 isolates), Gentamicin (34 isolates) and Ciprofloxacin (32 isolates). Least sensitivity was observed for Imipenem (18 isolates) Ceftazidime (18 isolates) and Piperacillin-Tazobactam (8 isolates). and Extended spectrum beta lactamase (ESBL) producing 6 strains of *Klebsiella pneumoniae* were noted by using Ceftazidime and Ceftazidime -Clavulanic acid discs.

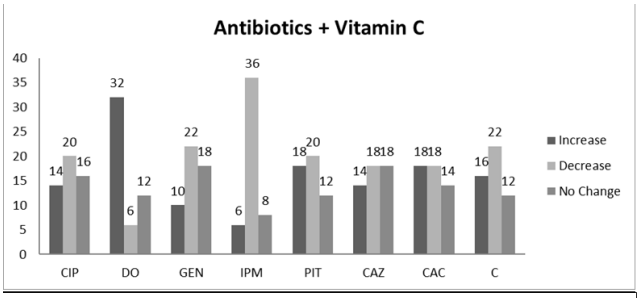



CIP-Ciprofloxacin; DO-Doxycycline; GEN-Gentamicin; IMP-Imipenem; PIT-Piperacillin tazobactam; CAZ-Ceftazidime; C-Chloramphenicol

**Fig. 1:** Showing Sensitivity pattern of *Klebsiella pneumoniae*.

### 3.1. Vitamin C and Antibiotics

Differences of about 1-6 mm change were observed in zone diameter on addition of Vitamin C to the antibiotics. The number of isolates showing increases, decreases and no change are depicted for each antibiotic in Figure 2.




CIP-Ciprofloxacin; DO-Doxycycline; GEN-Gentamicin; IMP-Imipenem; PIT-Piperacillin tazobactam; CAZ-Ceftazidime; CAC-Ceftazidime Clavulanic acid; C-Chloramphenicol

**Fig. 2:** Showing effect of Vitamin C on antibiotics.

On addition of Vitamin C to ciprofloxacin of the 50 isolates, 14 showed increase, 20 showed decrease and 16 did not show any change in zone diameter as observed with antibiotic discs alone. Out of the 32 isolates which were sensitive for Ciprofloxacin, on addition of Vitamin C solution, 12 isolates showed increase in zone, 16 isolates showed decrease of zone and 4 isolates did not show any change in zone. On addition of Vitamin C, out of the 14 resistant isolates, 2 isolates showed increase in zone, 2 isolates showed decrease in zone and 10 isolates showed no change in zone. The increase in zone ranged from 1-6mm and decrease in zone ranged from 1-6mm. The increase was not significant but the decrease was significant on analysis by student t test.

As per student t test analysis, significant increase in zone diameter were observed on addition of Vitamin C to antibiotics Doxycycline (1-6mm by 32 isolates) and Ceftazidime-Clavulanic acid (1-6mm by 18 isolates). Significant decrease in zone diameter was observed on addition of Vitamin C to antibiotics, Imipenem (2-14mm by 28 isolates), Ciprofloxacin (1-6mm by 20 isolates), and Ceftazidime-Clavulanic acid (2-10mm by 18 isolates).

On testing with Ceftazidime-Clavulanic acid and Ceftazidime, 6 ESBL isolates were noted out of which all 6 isolates showed increase in zone along with Vitamin C. Among the 44 non ESBL isolates, increase in zone was observed in 12 isolates, 14 isolates showed decrease in zone and 14 isolates showed no change in zone. The increase ranged from 1-6mm and decrease ranged from 2-10mm. Both the increase and decrease were significant on analysis by student t test. It is to be noted that effect of Vitamin C on Ceftazidime-Clavulanic acid is contradictory, as both increase and decrease in zone diameter were observed for equal number of isolates which were also statistically significant. No significant changes were observed with Gentamicin, Piperacillin tazobactam, Chloramphenicol and Ceftazidime. The results were noted in Table 1.

## 4. Discussion

With the growing population of drug resistant organisms and appearance of newer diseases, the mere survival of humans itself are difficult nowadays. *Klebsiella pneumoniae*, is an important bacterium causing respiratory infections and also a potent nosocomial pathogen. Over the last few decades, there has been a shift from sensitive K. pneumoniae to drug resistant strains such as extendedspectrum -lactamases (ESBLs), carbapenemases producers (AmpC) and Metallobetalactamase producers (MBL) by K. pneumonia.[15] The human immune system encounters innumerable number of pathogens and protects against the infection by producing various immune responses. The proper functioning of immune response depends of various essential elements. Due to decrease in ability of humans to synthesize various essential elements, the external supplementation is mandatory to regulate normal biological responses and to prevent deficiency diseases. Like that, Vitamin C supplementation is also one of the frequently prescribed therapy. The role of vitamin C as anti-oxidant, immunomodulator and collagen synthesis are well studied. Vitamin C is essential for both innate and adaptive immune responses. The potent antibacterial effects of vitamin C are, at least in part, due to its low pH and thus milieu-modifying properties.[16]

In this prospective study, we took the 50 isolates of *Klebsiella pneumoniae* from clinical samples. The antibiotic sensitivity was done with standard antibiotic drugs and the sensitivity pattern was noted. The percentage of sensitivity to antibiotics Chloramphenicol (84%), Doxycycline (68%), Gentamicin (68%), Ciprofloxacin (64%), Imipenam (36%) Ceftazidime (36%) and Piperacillin-Tazobactam (16%). It was noted that 6(12%) isolates out of 50 were ESBL producing, indicating presence of drug resistant isolates. In a study conducted in 2013, 23% of ESBL producing and 11% of carbapenemase producing *Klebsiella pneumoniae* were observed (Kleb).

Vitamin C solution 1 mg (10$\mu$l) was added to each antibiotic disc and the change in sensitivity pattern was noted. Differences of about 1-6 mm change were observed in zone diameter on addition of Vitamin C to the antibiotics. Vitamin C to ciprofloxacin - 14 showed increase, 20 showed decrease and 16 did not show any change in zone diameter. The increase was not significant but the decrease was significant on analysis by student t test. Significant increase in zone diameter were observed on addition of Vitamin C to antibiotics Doxycycline (1-6mm by 32 isolates) and Ceftazidime-Clavulanic acid (1-6mm by 18 isolates). Significant decrease in zone diameter was observed on addition of Vitamin C to antibiotics, Imipenem (2-14mm by 28 isolates), Ciprofloxacin (1-6mm by 20

**Table 1:** Vitamin C and Antibiotics

| Antibiotic | Isolates/50 | Vitamin C | | | | | |
|---|---|---|---|---|---|---|---|
| | | Increase | | Decrease | | Same | |
| Ciprofloxacin | Sensitive-32<br>Intermed-4<br>Resistant-14 | 12<br>-<br>2 | 1-6mm Not Significant | 16<br>2<br>2 | 1-6mm Significant | 4<br>2<br>10 | Not Significant |
| Doxycycline | Sensitive-34<br>Intermed-4<br>Resistant-12 | 22<br>2<br>8 | 1-6mm Significant | 4<br>2<br>- | 1-4mm Not Significant | 8<br>-<br>4 | Not Significant |
| Gentamicin | Sensitive-34<br>Intermed-2<br>Resistant-14 | 4<br>2<br>4 | 1-10mm Not Significant | 16<br>-<br>6 | 1-6mm Not Significant | 14<br>-<br>4 | Not Significant |
| Imipenem | Sensitive-18<br>Intermed-20<br>Resistant-12 | -<br>12<br>2 | 2-5mm Not Significant | 18<br>4<br>6 | 2-14mm Significant | -<br>4<br>4 | Not Significant |
| PIT | Sensitive-8<br>Intermed-20<br>Resistant-22 | 4<br>6<br>8 | 2-8mm Not Significant | 4<br>4<br>12 | 1-9mm Not Significant | -<br>10<br>2 | Not Significant |
| Ceftazidime | Sensitive-18<br>Intermed-8<br>Resistant-24 | 8<br>6<br>- | 1-4mm Not Significant | 10<br>2<br>6 | 1-15mm Not Significant | -<br>-<br>18 | Not Significant |
| Ceftazidime Clavulanic acid | ESBL-6<br>NON ESBL-44 | 6<br>12 | 1-6mm Significant | -<br>18 | 2-10mm Significant | -<br>14 | Not Significant |
| Chloramphe-nicol | Sensitive-42<br>Intermed-4<br>Resistant-4 | 14<br>-<br>2 | 1-4mm NOT Significant | 20<br>-<br>2 | 1-10mm Not Significant | 8<br>4<br>- | Not Significant |

isolates), and Ceftazidime-Clavulanic acid (2-10mm by 18 isolates). On testing with Ceftazidime-Clavulanic acid and Ceftazidime, 6 ESBL isolates were noted out of which all 6 isolates showed increase in zone along with Vitamin C.No significant changes were observed with Gentamicin, Piperacillin tazobactam, Chloramphenicol and Ceftazidime.

Pneumonia is the most common severe infection, which is usually caused by bacteria and viruses. Three controlled studies recorded a reduction of at least 80% in the incidence of pneumonia when supplemented with vitamin C.[17,18] Many studies shows potent inhibitory action of Vitamin C either alone or in combination with other natural extracts.[19] against bacterial,[20] viral,[21] fungal[22] and parasitic organisms.[23] In the study conducted by Wang Y and Jia et al[24] they documented that Vitamin C decreases the antifungal activity of Fluconazole against Candida species in murine models . Similarly in our study, it was noted that Vitamin C significantly decreased the inhibition zone of antibiotics Ciprofloxacin (40%), Imipenem (72%) and Ceftazidime-Clavulanic acid (36%) indicating a decrease in their antibacterial effect. Also Vitamin C enhanced the antibacterial effect of Doxycycline (64%) and Ceftazidime-Clavulanic acid (36%) significantly. It is to be noted that effect of Vitamin C on Ceftazidime-Clavulanic acid is contradictory, as both increase and decrease in zone diameter were observed for equal number of isolates which were also statistically significant. This might be due to small sample size of isolates (Number – 50) undertaken in the study.

The observations noted in this study need further development in terms of increased number of isolates and proper standardization of Vitamin concentrations, with respect to each antibiotic. Being an invitro study, it does not establish any chemical interactions between Vitamins and antibiotics and needs further research by clinical methods. Studies can be done to compare the effect of Vitamins on antibiotics against other microorganisms also. The standardization of Vitamins would be essential to document the interactions based on Minimum inhibitory concentration of each antibiotic.

## 5. Conflict of Interest

The authors declare that there are no conflicts of interest in this paper.


## 6. Source of Funding

None.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