How to Cite:
Setiawan, A., Agung, D. W. W., & Muhammad, V. A. (2022). Comparison dose effects of vitamin C and zinc administration to inhibit klebsiella pneumoniae biofilms formation. *International Journal of Health Sciences*, *6*(S9), 328–335. https://doi.org/10.53730/ijhs.v6nS9.12262

Comparison dose effects of *vitamin* C and *zinc* administration to inhibit *klebsiella pneumoniae* biofilms formation

Andy Setiawan
Department of Clinical Microbiology, Faculty of Medicine, Airlangga University/RSUD Dr. Soetomo, Surabaya, Indonesia
Corresponding authors email: crazy07doctor@yahoo.com

Agung Dwi W. W.
Department of Clinical Microbiology, Faculty of Medicine, Airlangga University/RSUD Dr. Soetomo, Surabaya, Indonesia

Muhammad Vitanata A.
Department of Clinical Microbiology, Faculty of Medicine, Airlangga University/RSUD Dr. Soetomo, Surabaya, Indonesia

Abstract*---Background*: *Klebsiella pneumoniae* is a Gram-negative bacterium that form biofilm and causes various infections. Biofilms have important role in resistance to antibiotics. Alternative agents that can inhibit biofilms formation with minimal side effects is required. This study show effects of vitamin C and zinc to inhibit *Klebsiella pneumoniae* biofilms formation. *Method*: This experiment used clinically stored *Klebsiella pneumoniae* isolates using controls. Isolates other than control are exposed with vitamin C and zinc oral preparations with various doses invitro then Optical Density and percentage reduction in biofilm is calculated and compared. *Results*: Vitamin C and zinc had invitro-inhibiting effect on biofilms formation of *Klebsiella pneumoniae* at all doses. The differences in decrease of Optical Density biofilm from the doses used after statistical testing was significant ($P < 0.05$). The smallest Optical Density and the largest percentage of Optical Density reduction of biofilms is found in vitamin C 1000 mg and zinc 50 mg. *Conclusion*: There are differences in inhibition of biofilm formation *Klebsiella pneumoniae* in the administration of vitamin C and zinc, with higher dose of vitamin C and zinc, the inhibition of biofilms formation is greater.

***Keywords*---**Vitamin C, zinc, biofilms, *Klebsiella pneumoniae*, Optical Density.

Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is a negative Gram bacterium that can cause bacteremia and sepsis, urinary tract infections, pneumonia, and nosocomial infections (Ashurst and Dawson, 2018). *K. pneumoniae* is known to have the ability to form biofilms, which are communities of bacteria attached to the extracellular matrix. Biofilms play an important role in bacterial virulence because they can avoid phagocytosis and bacteria become more resistant to antibiotics (Nirwati, H *et al.*, 2019). Antibiotics are used to inhibit biofilms, but their use can cause Multiple drug resistance organisms (MDRO) and interfere with the normal flora of the body, so alternative agents are needed that have an effect on biofilms with minimal side effects (Llor, C and Bjerrum, L, 2014).

The prevalence of *K. pneumoniae* infection is 13% in America, 5% in Pakistan, 64.2% in Nigeria, 33.9% in India, 17.4% in Denmark, and 14.1% in Singapura (Virawan, Nuryastuti, and Nirwati, 2020). Studies in Klaten in 2017, 148 (85.63%) of 167 isolates of *K. pneumoniae* formed biofilms (Nirwati, H *et al.*, 2019). A 2016 study in Iran showed the majority of *K. pneumoniae* (93.6%) formed biofilms. (Seifi *et al.*, 2016).

Vitamin C and zinc micronutrients that are very important and have many roles in the body (Gombart, A. F *et al.*, 2020). Vitamin C and zinc are useful for immune support, have antimicrobial effects including on *K.pneumoniae* and have the effect of inhibiting the formation of biofilms (Gombart, A. F *et al.* , 2020; McDevitt *et al.*, 2011).

Studies on oral supplementation of vitamin C and zinc and the inhibitory effect on the formation of biofilms invitro are still limited, so this study wants to show and compare the effects of vitamins C and oral zinc in various doses invitro to inhibit the formation of biofilms in isolates stored from *K. pneumoniae* in the Clinical Microbiology Unit of Dr. Soetomo Hospital.

Methods

Study design

This type of research is true experimental laboratory**.** The design of this study is a post testonly control group design, experimental research with a control group, data taken after treatment.

Study participants

The research population is clinical isolates stored from *K. pneumoniae* at Dr. Soetomo Hospital. The research sample was a clinical isolate stored from *K. pneumoniae* which formed a biofilm in the Clinical Microbiology Unit of Dr. Soetomo Hospital. The magnitude of the sampel used in clinical isolates stored from *K. pneumoniae* was 6 with 4 replications. Research sampling is by *random* sampling.

Study place and time

This research was conducted at the Clinical Microbiology Unit of Dr. Soetomo Hospital, Surabaya in June 2022.

Study material and instruments

K. pneumoniae isolates, vitamin C oral preparations, zinc sulphate oral preparations. Consumables include kristal violet 0.1%, aquedes, nutrient agar, *Tryptic Soy Broth* (TSB), methanol, 95% ethanol, *Phosphate Buffer Saline* (PBS), sucrose 5%, sterile cotton swabs, gloves, masks.

Preliminary study

Preliminary tests were conducted to determine the ability of clinical isolates of *K. pneumoniae* in the formation of biofilms. Clinical isolates of *K. pneumoniae* were isolated and cultured for 24 hours then 0.5 McFarland suspension was made in TSB media and 5% sucrose was added then inserted into the microtiter plate. Afte incubating for 24 hours, the substrate is removed, washed with PBS (3x), fixed methanol and stained with violet crystals. The formed biofilm is observed its density.

Study plot

Clinical isolates stored of *Klebsiella pneumoniae* planted in NA and incubation of 35±2 ºC for 24 hours, take 3 – 5 colonies put in TSB then vortex, with nephelometer adjust concentration to 0.5 McF ($1x10^8$ CFU/ml), add 5% sucrose. Vitamin C 250, 500,750,1000 mg and zinc 10, 20, 40, 50 mg Each dose is dissolved in NS. The microwell plate is filled with 180 µl of TSB and 20 µl of bacterial suspension for control. The microwell plate was filled with 180 µl of TSB and 20 µl of bacterial suspension with plus 20 µl of vitamin C solution of each concentration. The microwell plate was filled with 180 µl of TSB and 20 µl of bacterial suspension with plus 20 µl of zinc solution of each concentration. Incubation 35±2 ºC for 24 hours. The contents of the microtiter plate are removed, washed with 300 µl PBS 3x, dried in reverse position. Fixation with 150 µl of methanol in each well, let stand 20 minutes, then the microplate is emptied by tapping and left in an upside-down position. It is stained with 0.1% violet crystals for 15 minutes. The microplate is drained, pour 300 µl of ethanol in each well for destaining for 15 minutes. The biofilm formed was measured by ELISA reader. Collection of measurement results and make graphs.

Data collection and analysis approach

Clinical isolates stored of *K. pneumoniae* observed the formation of its biofilm after being exposed to vitamin *C* and zinc at various doses. Data collection was carried out by measuring optical density (OD) using an ELISA reader. Data analysis was carried out with SPSS 26 using statistical method one-way ANOVA which aims to determine the significance of the effect of vitamin C and zinc administration at various doses on inhibition of formation biofilm, then created a graph using GraphPad Prism version 8.

Discussion

Data Results

This study aims to analyze the effect of vitamin C inhibition at a dose of 250 mg, 500 mg, 750 mg, 1000 mg and *zinc* at a dose of 10 mg, 20 mg, 40 mg, 50 mg on the formation of biofilm *K. pneumoniae* invitro. Clinical isolates of *K. pneumoniae* in subcultures on *nutrient agar* media for 1x24 hours at a temperature of 35±2

ºC. Before the study was carried out, a preliminary test was carried out to find out whether the isolates formed a biofilm.

Table 1 Data on the mean OD of *K. pneumoniae* biofilm against the administration of *vitamin* C and *zinc*

Isolate	*Klebsiella pneumoniae* (mean OD)									
	Vitamin C				*Zinc*				Positive control	Negative control
	250mg	500mg	750mg	1000mg	10mg	20mg	40mg	50mg		
1	0,225	0,170	0,111	0,072	0,254	0,150	0,094	0,064	0,311	0,048
2	0,251	0,189	0,092	0,056	0,230	0,199	0,072	0,055	0,270	0,048
3	0,264	0,158	0,123	0,054	0,249	0,217	0,083	0,054	0,319	0,048
4	0,235	0,165	0,099	0,052	0,218	0,176	0,097	0,075	0,313	0,048
5	0,212	0,147	0,087	0,064	0,193	0,165	0,088	0,077	0,280	0,048
6	0,243	0,190	0,125	0,068	0,233	0,203	0,090	0,069	0,320	0,048

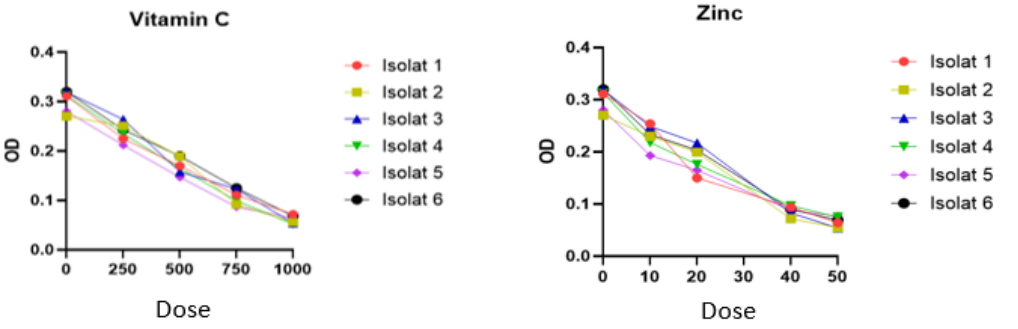

Figure 1 Graph of mean OD biofilm *K. pneumoniae* against administration of *vitamin* C and *zinc*

Table 2 Mean data on the decrease in the percentage of OD biofilm *K. pneumoniae* against the administration of *vitamin* C and *zinc*

Isolate	*Klebsiella pneumoniae* (mean percent)									
	Vitamin C				*Zinc*				Positive control	Negative control
	250mg	500mg	750mg	1000mg	10mg	20mg	40mg	50mg		
1	27,65	45,34	64,31	76,85	18,33	51,77	69,77	79,42	0	0,048
2	7,04	30,00	65,93	79,26	14,84	26,30	73,33	79,63	0	0,048
3	17,24	50,47	61,44	83,07	21,94	31,97	73,98	83,07	0	0,048
4	24,92	47,28	68,37	83,39	30,35	43,77	69,01	76,04	0	0,048
5	24,29	47,50	68,93	77,14	31,07	41,07	68,57	72,50	0	0,048
6	24,06	40,66	60,94	78,75	27,19	36,56	71,87	78,44	0	0,048

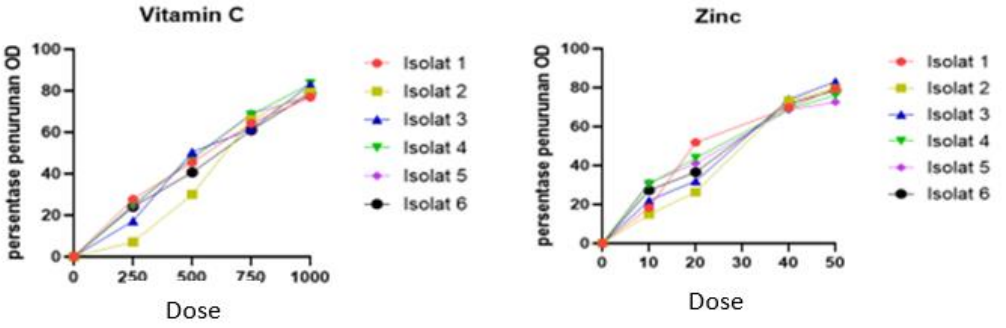

Figure 2 Graph of the mean decrease in the percentage of OD biofilm *K. pneumoniae* against the administration of *vitamin* C and *zinc*

Table 3 Significance of differences between normally distributed doses of *vitamin* C with ANOVA followed by *Post – Hoc* tests using LSD

Vitamin C dose	250 mg	500 mg	750 mg	1000 mg
250 mg	-	0,000	0,000	0,000
500 mg	0,000	-	0,000	0,000
750 mg	0,000	0,000	-	0,000
1000 mg	0,000	0,000	0,000	-

Table 4 Significance of differences between normally distributed zinc doses and the *Kruskal – Wallis* test followed by the *Mann – Whitney* test

Zinc dose	10 mg	20 mg	40 mg	50 mg
10 mg	-	0,016	0,004	0,004
20 mg	0,016	-	0,004	0,004
40 mg	0,004	0,004	-	0,01
50 mg	0,004	0,004	0,01	-

Results Discussion

The formation of biofilms is an important characteristic of the *Enterobacteriaceae* group including *K. pneumoniae* which is often an important virulence factor (Ramos-Vivas *et al.*, 2019). Antibiotics are used to inhibit biofilms, but their use can cause Multiple drug resistance organisms (MDRO) and interfere with the normal flora of the body, so alternative agents are needed that have an effect on biofilms with minimal side effects (Llor, C., & Bjerrum, L, 2014). Vitamin C and zinc at varying doses have the effect of inhibiting the formation of biofilms (Gombart, A. F, Pierre, A, and Maggini, S, 2020; McDevitt *et al.*, 2011). This study compared the effects of several doses of vitamin C and zinc oral supplements to inhibit the formation of biofilm *K. pneumoniae* invitro.

Administration of vitamin C at a dose of 250 mg, 500 mg, 750 mg, 1000 mg invitro at biofilm 6 clinical isolates *K. pneumoniae*, all showed the presence of a decrease in OD compared to the positive control group and after statistical testing, the difference in the decrease in OD biofilm from the dose used was all significant. The percentage decrease in biofilm in 6 clinical isolates of *K.pneumoniae* compared to positive controls on the administration of vitamin C at doses of 250 mg, 500 mg, 750 mg, 1000 mg invitro all increased along with the increase administered dose.

There are studies that invitro vitamin C has no effect on the growth of opportunistic bacteria such as *K. pneumoniae* but has the effect of inhibiting the formation of biofilms. The results of this study are the same as the study which also shows that the greater the dose of vitamin C given, the inhibitory effect on the biofilm is also greater (Kallio *et al.*, 2012; Khameneh *et al.*, 2016; Helgadóttir *et al.*, 2017; Pandit *et al.*, 2017).

Vitamin C when the dose is increased will be more pro-oxidant. *Vitamin* C increases oxidative stress from increased ROS production and then causes cell viability to be lost and interferes with metabolic activity, cell signaling (quorum sensing), EPS production, leakage of intracellular proteins, sugars, and down regulating biofilm gene expression from bacteria. *Quorum sensing* is the communication of intercellular bacteria where small chemical molecules (called *auto inducers*, for example such as *acyl homoserine lactones* (AHL) for Gram-negative bacteria) produced and in *sense* that causes a cascade of reactions that regulate many genes and are responsible for the expression of virulence factors, he formation of biofilms (Pandit *et al.*, 2017).

Administration of zinc at a dose of 10 mg, 20 mg, 40 mg, 50 mg invitro at biofilm 6 clinical isolates *K.pneumoniae*, all showed the presence of a decrease in OD compared to the positive control group and after statistical testing, the difference in the decrease in OD biofilm from the dose used was all significant. The percentage of biofilm reduction in 6 clinical isolates of *K.pneumoniae* compared to positive controls on zinc administration at doses of 10 mg, 20 mg, 40 mg, 50 mg invitro all increased along with dose increases a given.

There are studies that invitro zinc has the effect of inhibiting the formation of biofilms from *K.pneumoniae*. The results of this study are the same as the study which also shows that the greater the dose of zinc given, the inhibitory effect on the biofilm is also greater as evidenced by the the percentage of reduction in biofilm formation is getting bigger as the dose of zinc administered increases (Sharma *et al.*, 2022).

Zinc can interact with the components of the matrix. eDNA is a component that exists in almost all biofilm matrices and can function as a zinc chelator and such interactions have an impact on the stability of the biofilm. Zinc also interferes with other cellular mechanisms such as signaling and gene regulation (quorum sensing). Zinc can be bound to a ferric uptake regulator and affect iron homeostasis. Zinc also inhibits the cyclic diguanylate phosphodiesterase pathway which plays a role in biofilm regulation so that zinc will interfere with the biofilm formation process (Wu *et al*, 2013).

Vitamin C at a dose of 1000 mg on average causes a decrease in biofilm OD more and the percentage decrease in biofilm OD is higher than zinc at a dose of 50 mg
.

Conclusion

There are differences ininhibition of the formation of biofilm *K. pneumoniae* in the administration of *vitamin* C at doses of 250 mg, 500 mg, 750 mg, 1000 mg and *zinc* at doses of 10 mg, 20 mg, 40 mg, 50 mg invitro, where the higher the dose of *vitamin* C and *zinc* inhibition against the formation of biofilm *K. pneumoniae* is getting bigger and bigger. Studies inhibiting the formation of biofilm *K. pneumoniae* are still rare to use oral supplements, so it is necessary to conduct further studies using other vitamins or minerals so that there are more and more alternative agents other than antibiotics to inhibit the formation of biofilms. In this study, *vitamin* C and *zinc* can inhibit the formation of *K. pneumoniae* biofilm invitro, further studies are needed to see this effect on invivo.

Acknowledgements
I am grateful to Agung Dwi W W and Muhammad Vitanata A as my mentors and to my friends Tiar S.U.S, Agung D.S, Erizka R, Eny P who help me to finish this study.

References

Ashurst, J. V., & Dawson, A. (2018). 'Klebsiella pneumoniae'.

Gombart, A. F., Pierre, A., & Maggini, S. (2020). A review of micronutrients and the immune system–working in harmony to reduce the risk of infection. *Nutrients*, *12*(1), 236.

Helgadóttir, S., Pandit, S., Mokkapati, V. R., Westerlund, F., Apell, P., & Mijakovic, I. (2017). Vitamin C pretreatment enhances the antibacterial effect of cold atmospheric plasma. *Frontiers in cellular and infection microbiology*, *7*, 43.

Kallio, J., Jaakkola, M., Mäki, M., Kilpeläinen, P., & Virtanen, V. (2012). Vitamin C inhibits staphylococcus aureus growth and enhances the inhibitory effect of quercetin on growth of Escherichia coli in vitro. *Planta medica*, *78*(17), 1824-1830.

Khameneh, B., Bazzaz, B. S. F., Amani, A., Rostami, J., & Vahdati-Mashhadian, N. (2016). Combination of anti-tuberculosis drugs with vitamin C or NAC against different Staphylococcus aureus and Mycobacterium tuberculosis strains. *Microbial pathogenesis*, *93*, 83-87.

Llor, C., & Bjerrum, L. (2014). Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. *Therapeutic advances in drug safety*, *5*(6), 229-241.

McDevitt, C. A., Ogunniyi, A. D., Valkov, E., Lawrence, M. C., Kobe, B., McEwan, A. G., & Paton, J. C. (2011). A molecular mechanism for bacterial susceptibility to zinc. *PLoS pathogens*, *7*(11), e1002357.

Nirwati, H., Sinanjung, K., Fahrunissa, F., Wijaya, F., Napitupulu, S., Hati, V. P., & Nuryastuti, T. (2019, December). Biofilm formation and antibiotic resistance of Klebsiella pneumoniae isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. In *BMC proceedings* (Vol. 13, No. 11, pp. 1-8). BioMed Central.

Pandit, S., Ravikumar, V., Abdel-Haleem, A. M., Derouiche, A., Mokkapati, V. R. S. S., Sihlbom, C., ... & Mijakovic, I. (2017). Low concentrations of vitamin C reduce the synthesis of extracellular polymers and destabilize bacterial biofilms. *Frontiers in microbiology*, *8*, 2599.

Ramos-Vivas, J., Chapartegui-González, I., Fernández-Martínez, M., González-Rico, C., Fortún, J., Escudero, R., ... & Fariñas, M. C. (2019). Biofilm formation by multidrug resistant Enterobacteriaceae strains isolated from solid organ transplant recipients. *Scientific reports*, *9*(1), 1-10.

Seifi, K., Kazemian, H., Heidari, H., Rezagholizadeh, F., Saee, Y., Shirvani, F., & Houri, H. (2016). Evaluation of biofilm formation among Klebsiella pneumoniae isolates and molecular characterization by ERIC-PCR. *Jundishapur journal of microbiology*, *9*(1).

Sharma, R. P., Raut, S. D., Jadhav, V. V., Mulani, R. M., Kadam, A. S., & Mane, R. S. (2022). Assessment of antibacterial and anti-biofilm effects of zinc ferrite nanoparticles against Klebsiella pneumoniae. *Folia Microbiologica*, 1-9.

Suryasa, I. W., Rodríguez-Gámez, M., & Koldoris, T. (2021). Get vaccinated when it is your turn and follow the local guidelines. International Journal of Health Sciences, 5(3), x-xv. https://doi.org/10.53730/ijhs.v5n3.2938

Virawan, H., Nuryastuti, T., & Nirwati, H. (2020). Indonesian Journal of Medicine and Health.

Wido, A., Bajamal, A. H., Apriawan, T., Parenrengi, M. A., & Al Fauzi, A. (2022). Deep vein thrombosis prophylaxis use in traumatic brain injury patients in tropical climate. International Journal of Health & Medical Sciences, 5(1), 67-74. https://doi.org/10.21744/ijhms.v5n1.1840

Wu, C., Labrie, J., Tremblay, Y. D. N., Haine, D., Mourez, M., & Jacques, M. (2013). Zinc as an agent for the prevention of biofilm formation by pathogenic bacteria. *Journal of applied microbiology*, *115*(1), 30-40.