Original Research Article

Activities of Macrolides in Combination with Vitamin D against *Bacillus cereus* Isolated from Soil of a Lanfill Site in Nsukka Southeast Nigeria

ABSTRACT

Background: The fast moving world of antibiotics resistance is now a big concern, and our antibiotics are in danger of extinction. This is concern to us because; an empty 'antibiotic pipeline' leads to devastating future.

Aim: The aim of this study was to isolate *Bacillus cereus* from soil contaminated with various toxic and domestic wastes and investigate its sensitivity to macrolide antibiotics (erythromycin ERY and azithromycin AZM) in combination with vitamin D supplement.

Methodology: Five soil samples were collected from a landfill site and serially diluted up to 10^{-6}. *B. cereus* was isolated from the soil sample using Mannitol egg Yolk Polymyxin agar medium (MYP), a selective nutrient medium for *B. cereus* isolation. The sensitivity of *B. cereus* to erythromycin and azithromycin was screened in Mueller-Hinton agar (MHA) containing varying concentrations (5, 10, 20 mg/ml) of vitamin D. Agar without vitamin D (0.00mg/ml) served as the control. The activities of ERY and AZM in combination with vitamin D were determined by measuring the zones of inhibition.

Result: *In vitro* antibiotic susceptibility tests on our isolate showed maximum sensitivity to ERY and maximum resistance to AZM with 0.00mg/ml (no combination) of vitamin D. Contrarily, ERY was found to be least active, with the zones of inhibitions decreasing from 28mm to 16mm, with increasing concentration of vitamin D. On the other hand, AZM achieved maximum sensitivity from 12mm to 25mm, with increasing concentration of vitamin D.

Conclusion: The activities appeared to be important but dose-dependent. So, the systematic use of Vitamin D in combination with macrolide antibiotics may be efficient in treating food and soil infections caused *B. cereus.* However, *in vivo* evaluation of these activities is needed.

Key words: *Erythromycin; Azithromycin; Vitamin D; Bacillus cereus; Landfill; Soil*

1. INTRODUCTION

Bacillus cereus is a soil dweller that thrives in diversity of habitats or as a part of the intestinal flora of different animals. The ability of *B. cereus* to form endospores that are resistant to heat, dehydration and other physical stresses, enhances it's potential to withstand time and harsh environments [1]. On entry into mammalian tissues, *B. cereus* becomes an opportunistic pathogen that may cause severe local or systemic infections such as endophthalmitis and septicaemia [1,2]. When present in food or the gastrointestinal tract and it is one of the major foodborne pathogenic bacteria, although in most cases disease is mild and of short duration [2,3]. Interestingly, the spectrum of potential *B. cereus* toxicity ranges from strains used as probiotics for humans [4] to highly toxic strains reported to be responsible for food-related fatalities [5,6].

B. cereus causes two types of gastrointestinal disease, the diarrhoeal and the emetic syndromes, which are caused by very different types of toxins. The emetic toxin, causing vomiting, has been characterized and is a small ring-formed peptide [7], while the diarrhoeal disease is caused by one or more protein enterotoxins, thought to elicit diarrhoea by disrupting the integrity of the plasma membrane of epithelial cells in the small intestine [8]. The three toxins that have been implicated as aetiological agents of the diarrhoeal disease are the poreforming cytotoxins haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (CytK) [5,8,9]. Hbl and Nhe are homologous three-component toxins, which appear to be related to the monooligomeric toxin cytolysin A found in *Escherichia coli* [10].

Antibiotic treatment is still the major method for treating bacterial infections, including those caused by *B.* cereus. However, the wide use of antimicrobials has led to the emergence of antibiotic-resistant strains, including those resistant to multiple antibiotics, which are responsible for the failure of routine treatments [11]. Thus, it is paramount to determine the antibiotic resistance profile of *B. cereus* so as to make better selection for treatment regimens [12]. This emergency of antibiotic resistant strains of *B. cereus* has made treating these infections more challenging in recent times. The phenomenon has motivated researchers to develop various semi synthetic antibiotics, with the aim of curbing the effects of antibiotic resistance, but microbes also gained resistance against these semi synthetic antibiotics along with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions [13].

Food poisoning caused by *B. cereus* toxins is more prevalent in communities with poor waste management sanitation units and such infections are becoming difficult to treat and in some cases fatal. The emerging picture from literatures and reported trails shows that vitamin D has a potential antimicrobial activity and its deficiency has detrimental effects on general well-being and longevity [14,15]. Also, multiple mechanisms through which Vitamin D reduces the susceptibility to infection have been reported. Vitamin D also boosts innate immunity by modulating production of anti-microbial peptides (AMPs) and cytokine response [15]. These supplements are not only widely available but also cheap. Nonetheless, it has been reported that consumption of Vitamins or any other supplements with antibiotics sometimes decreases the antibacterial activity of many antibiotics [16]. These veritable findings gave impetus to this study. So, this has prompted us to isolate a bacterium from contaminated soil collected from a dumping ground and evaluate the *in vitro* activity of erythromycin and azithromycin in combination with Vitamin D.

2. MATERIALS AND METHODS

2.1 Sampling Area

The sampling area, Nsukka (6.84^0N and 7.37^0E), has urban and rural settings and is the site of the University of Nigeria, the first indigenous Nigerian university and one of Nigeria's largest universities. Nsukka is a town and Local Government Area in Enugu state, South-East, Nigeria.

2.2 Sample Collection

A total of five (5) Soil samples were collected from a landfill site in Nsukka in the month of July, 2019. The collection process was done aseptically from the surface layer (0-18cm). The temperature of the region during the time of collection was 31-32ºC and relative humidity was recorded as 39-45%. Other physical parameters like pH and Electrical Conductivity (EC) were also meticulously measured [17].

2.3 Isolation of Bacteria

The soil samples were serially diluted using 10 fold dilution up to 10^{-6} [18]. The serially diluted soil samples were poured on a Mannitol egg Yolk Polymyxin agar medium (MYP), a selective nutrient medium for the isolation of *B. cereus*. All plates were incubated for 24 hours at 37^0C. After incubation, typical colonies were pink-orange surrounded by a zone of precipitation indicating lecithinase production. These pink-orange colonies were presumptively identified to be *B. cereus*. Furthermore, typical colony presumed to belong to the *B. cereus* group was subcultured on nutrient agar slants and stored at 4°C. Subculture was made for every 30 days to maintain viability and purity.

2.4 Determination of the activity of erythromycin in combination with Vitamin D against Of *B. cereus*

The activity of erythromycin in combination with Vitamin D against *B. cereus* was determined using agar diffusion method [19] by following the principle of Kirby Bauer 1959 [20]. Three (3) sets of freshly prepared 18ml Mueller-Hinton agar (MHA) were supplemented separately with Vitamin D, such that the final concentrations were: 5mg/ml, 10mg/ml and 20mg/ml respectively. Equally, a set of 18ml MHA without Vitamin D served as control. The plates containing the various concentrations of the supplement were inoculated with an 18h old standardized inoculum of *B. cereus*, by carefully streaking with sterile cotton tipped swab to achieve confluent growth. The inoculated plates were then incubated at 37^oC overnight, under aerobic conditions. After incubation, the diameter of the zones of inhibition were 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 Analysis System (SAS) software version 9.3 (SAS, 2012). Significance was accepted at $P \leq 0.05$.

3. RESULTS

3.1 Measurement of Physical Parameters of Soil Sample

The physical properties of the soil (Table 1) are very much responsible for the kind of microorganism present. So the physical properties of the soil such as soil type, texture, pH and electrical conductivity was measured properly.

Table1: Physical parameters of Soil Sample

Soil Type	Texture	pH	Electrical Conductivity (μs/cm)
Clay	Granular	6.9	224.0

3.2 Activities of macrolides in combination with Vitamin D

The susceptibility (Table 2) of *B. cereus* towards erythromycin and Azithromycin in combination with Vitamin D was determined by measuring the diameter of the zone of inhibitions. Whether the antibiotics were susceptible was determined by antimicrobial breakpoints of *B. cereus* in CLSI's guideline [21]. Our isolate was sensitivity to erythromycin, but resistant to Azithromycin without combination of viatamin D ($p<0.05$). In contrast to Erythromycin, appreciable effects were witnessed in the activities of Azithromycin in combination with vitamin D.

Table2: Activity of macrolides in combination with Vitamin D

	Zone of inhibition (mm)			
Vitamin D	**0.00(mg/ml)**	**5(mg/ml)**	**10(mg/ml)**	**20(mg/ml)**
Erythromycin	28(**S**)	26(**S**)	16(**I**)	16(**I**)
Azithromycin	12(**R**)	17(**I**)	17(**I**)	25(**S**)

Key: ***0.00mg/ml =*** *control (without Vitamin D),* ***S =*** *Sensitive,* ***I =*** *Intermediate,* ***R =*** *Resistant*

4. DISCUSSION

In today's world, treating a disease caused by infectious agents of soil is a major problem, due to antibiotic resistance [22]. However, containment strategies are continually being thought of and evaluated. Beyond food poisoning, *B. cereus* is also associated with non-gastrointestinal infections and antibiotic susceptibility testing can provide a reference for the clinical treatment of food poisoning [12,23]. Supplements like Vitamin D, are now used in combination with some antibiotics as alternative clinical option for treating these diseases [24]. In our investigation, soil from landfill dump showed the presence of *Bacillus cereus* strain. This may be due to fact that Gram positive *Bacillus* sp survives in the pH (6-7) and our soil sample pH was 6.9. However, the results of this study showed depreciable and appreciable activities in the zones of inhibition of erythromycin and azithromycin respectively, against *B. cereus* in Mueller-Hinton agar containing Vitamin D, as compared to the control.

In this investigation, unfortunately, increasing concentration of Vitamin D greatly decreased the *in vitro* efficiency of erythromycin against *B. cereus*. Erythromycin produced zones of inhibition with maximum sensitive at 0.00mg/ml and lowest sensitive at 20mg/ml ($p<0.05$). The isolated *B. cereus* became resistant to erythromycin when combined with 10mg/ml and 20mg/ml of Vitamin D ($p<0.05$). On the contrary, the test organism was still susceptible to erythromycin when combined with 5mg/ml of Vitamin D ($p<0.05$). Even though the test organism was resistant to azithromycin, our study witnessed an elevated sensitivity to azithromycin with increasing concentrations of vitamin D ($p<0.05$). The study of Biswas et al. [25] witnessed appreciable results on *Bacillus* spp. However, their study used a different supplement, Vitamin C, from the supplement used in our study. The effects witnessed in our study may be due to the fact that Vitamin D reduced the oxidative stress provided by the antibiotics which inversely helped protect the pathogen. This may be the reason why erythromycin showed decrease in the zone of inhibition on *B. cereus* when given in combination with Vitamin D.

The combination with vitamin D greatly enhanced the bacteriocidal potential of Azithromycin. Interestingly, emerging reports from literatures suggests macrolide antibiotics inhibit bacterial protein synthesis by binding to the 50s ribosomal subunit hence disrupting ribosomal RNA molecule and various ribosomal proteins [26]. The vitamin D used in this study may have influenced the binding of azithromycin to the 50s ribosomal subunit which resulted to increased effectiveness of azithromycin against our isolate. Many studies have evaluated the effects of some supplements on various bacteria species such as *K. pneumoniae and E. coli* [27], *S. aureus* [28], *Helicobacter pylori and Campylobacter jejuni* [29] and *Mycobacterium tuberculosis* [30], all studies reported significant effects. Hence, our investigation has shown that although erythromycin (30 µg) was efficient on *B. cereus* isolated from soil of a landfill site, its *in vitro* efficiency significantly decreased with increasing concentration of Vitamin D. However, azithromycin (15 µg) showed increased bactericidal potential with increasing concentration of Vitamin D.

5. CONCLUSION

It is seen from this investigation that macrolide antibiotics in combination with vitamin D exerted a concentration-dependent activity on *B. cereus* isolated from landfill site. Varying concentrations of the vitamin D had significant effects by decreasing the zones of inhibition of the erythromycin on the test organism, but increased the zones of inhibition of azithromycin. These findings may serve as guild during antimicrobial therapy for treatment of notorious multidrug resistant infections. Lastly, further works are also needed to study other antibiotics efficacy in combination with vitamins against pathogens of clinical and environmental importance. There is also need to evaluate similar activities *in vivo*, using laboratory animals.

REFRENCES

1. Kotiranta A, Lounatmaa K, Haapasalo M. Epidemiology and pathogenesis of Bacillus cereus infections. Microbes Infect. 2000;2:189–198.
2. Pushpendra S, Rajesh S, Ashish KS, Ravindra S. Isolation of Bacillus spp. from Soil for Antimicrobial Production and Antibiotic Resistance. Adv Biotech Micro. 2018;8(4):555741.DOI: 10.19080/AIBM.2018.08.555741.
3. Marrollo R. “Bacillus cereus food-borne disease,” in The Diverse Faces of Bacillus cereus, ed. V. Savini (London: Academic Press), 2016;61–72.
4. Hong HA, Duc Le H, Cutting SM. The use of bacterial spore formers as probiotics. FEMS Microbiol Rev. 2005;29:813–835.
5. Lund T, De Buyser ML, Granum PE. A new cytotoxin from Bacillus cereus that may cause necrotic enteritis. Mol Microbiol. 2000;38:254–261.
6. Dierick K, Van Coillie E, Swiecicka I, Meyfroidt G, Devlieger H, Meulemans A, et al. Fatal family outbreak of Bacillus cereus associated food poisoning. J Clin Microbiol. 2005;43: 4277–4279.
7. Ehling-Schulz M, Fricker M, Scherer S. Bacillus cereus, the causative agent of an emetic type of food-borne illness. Mol Nutr Food Res. 2004;48:479–487.
8. Beecher DJ, MacMillan JD. Characterization of the components of hemolysin BL from Bacillus cereus. Infect Immun. 1991;59:1778–1784.
9. Lund T; Granum PE. Characterisation of a nonhaemolytic enterotoxin complex from Bacillus cereus isolated after a foodborne outbreak. FEMS Microbiol Lett. 1996;141:151–156.
10. Arnesen LPS, Fagerlund A, Granum PE. From soil to gut:Bacillus cereus and its food poisoning toxins. FEMS Microbiol Rev. 2008;(32):579–606
11. Friedman ND, Temkin E, Carmel Y. The negative impact of antibiotic resistance. Clin. Microbiol. Infect. 2016;22, 416–422.
12. Yu S, Yu P, Wang J, Li C, Guo H, Liu C, et al. A Study on Prevalence and Characterization of Bacillus cereus in Ready-to-Eat Foods in China. Front. Microbiol. 2020;10:3043. doi: 10.3389/fmicb.2019.03043
13. Smith H. Side-effects of broad-spectrum antibiotics. South African Pharmacist's Assistant, 2011;11(2):12.
14. Bikle D. Vitamin D and the immune system Curr Opin Nephrol Hypertens. 2008;17:348–352.

15. Youssef DA, Miller CT, El-Abbassi A, Cutchins DC, Cutchins C, Grant WB et al. Antimicrobial implications of vitamin D. Dermatoendocrinol. 2011;3(4):220-229.
16. Majed M, Mhaidat NM, Alzoubi KH, Al-Azzam SI, Shaweesh AI. Ciprofloxacin-Induced Antibacterial Activity is Reversed by Vitamin E and Vitamin C. Curr Microbiol, 2012;64(5):457-462.
17. Anderson-Cook CM, Alley MM, Roygard JK, Khosla R, Noble RB, Doolittle JA. Differentiating soil types using electromagnetic conductivity and crop yield maps. Soil Sci Soc of Am J. 2002;66:1562-1570.
18. Kaur M, Samyal M, Ahmed Z, Kaur A, Isolation characterization and identification of bacterial strain producing amylase. J Microbiol Biotech Res. 2012;2(4):573-579.
19. Boyan B, Hooper J, Parisot J. Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. Journal antimicrob chemother. 2008;61(6):1295-1301.
20. Bauer AW, Perry DM, Kirby WM. Single-Disk Antibiotic-Sensitivity Testing of Staphylococci An Analysis of Technique and Results. Arch Intern Med. 1959;104(2):208-216.
21. Clinical and Laboratory Standards Institute (CLSI). Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline, 2nd edn. M45A2E. Wayne: CLSI; 2010.
22. Chandan P, Mishra RP, Asif A, Gangwar VS, Shweta C. Isolation and Characterization of Multi drug Resistant Super Pathogens from soil Samples Collected from Hospitals. Res J Recent Sci. 2012;2:124-129.
23. Banerjee M, Nair GB, Ramamurthy T.Phenotypic& genetic characterization of Bacillus cereus isolated from the acute diarrhoeal patients. Indian J Med Res. 2011;133:8895.
24. Bikle DD. Vitamin D and the immune system: role in protection against bacterial infection. Curr Opin Nephrol Hypertens 2008;17:348–352.
25. Biswas S, Thomas N, Mandal A, Chandra D, Mukherjee S. Analysis of antimicrobial activity of vitamin C alone and in combination with antibiotics on Gram positive rod isolated from soil of a dumping site of Kolkata. Int J Pharm Biol Sci. 2013;3:101-110.
26. Lohsen S. Stephens DS. Current macrolide antibiotics and their mechanisms of action. Antibiotic Drug Resistance. 2019;97-117.
27. Verghese RJ, Mathew SK, David A. Antimicrobial activity of Vitamin C demonstrated on uropathogenic *Escheriachia coli* and *Klebsiella pneumoniae*. J Curr Res Sci Med. 2017;3*(*2):88-93.
28. Isela S, Sergio N, Jose M, Rene H, Claudio C. Ascorbic acid on oral microbial growth and biofilm formation. Pharm Inno*.* 2013;2:104-109.
29. Zhang HM, Wakisaka N, Maeda O, Yamamoto T. Vitamin C inhibits the growth of a bacterial risk factor gastric carcinoma: *Helicobacter pylori*. Cancer. 1997;80:1897-903.
30. Vilcheze C, Hartman T, Weinrick B, Jacobs WR. *Mycobacterium tuberculosis* is extraordinarily sensitive to killing by a vitamin C-induced Fenton reaction. Nat Comm. 2013;1:14-26.