Original Research Article

MOLECULAR DETECTION OF *Bacillus anthracis* FROM SOIL IN NIGER DELTA UNIVERSITY AND ENVIRONS, BAYELSA, NIGERIA

Abstract

Bacillus anthracis is an aerobic or facultative anaerobic, Gram-positive Bacilli which is naturally found in soil. *Bacillus anthracis* is known as the most dangerous bacterium which is used as a biological weapon causing cosmopolitan disease condition called anthrax infection. The study aimed at detecting *Bacillus anthracis* from Niger Delta University (NDU) Amassoma and her environs soil sample. Twenty soil specimens were collected randomly and processed, characterized by Microbiological and Polymerase Chain reaction technique. Disk diffusion method was used for Antimicrobial susceptibility testing. Findings from this study revealed that out of the 20 samples collected, 8(40%) were *Staphylococcus* species, 5(25%) were *Mucor* species while 7(35%) were *Bacillus* species, of the 7 positive for *Bacillus* 4(20%) had Mannitol fermentation while 3(15%) showed no fermentation, 3(15%) of the 7 also showed gamma hemolysis while 4(20%) showed complete (beta) hemolysis and 4(57.1%) showed turbidity while 3(15%) did not show turbidity. Antibiotic Susceptibility testing showed that Septrin, Ampicillin, Ceporex, Nalidixic acid and Gentamicin had total resistance to *Bacillus anthracis*, *Bacillus cereus* and other species. From the sequencing, other species of *Bacillus* were observed apart from *Bacillus anthracis*. This showed an advantage of genotypic identification over the phenotypic features. Hence proper identification of *Bacillus anthracis* from NDU and her environs soil sample must be put in place to create awareness for the students, staff, farmers, children and others who constantly have contact with the soil. Anthrax infection can also be controlled by improved personal hygiene.

Key words: *Bacillus anthracis*, soil sample, Niger Delta University, Antibiotics, environs

INTRODUCTION

Bacillus anthracis, a Gram-positive spore forming rod is the causative agent of anthrax and one of the organisms classified as biological warfare agents. Spores develops within the host to produce vegetative forms, which increase and present their virulence factors, destroying the host.

In case of outbreak or any intentional release, rapid and sensitive detection is essential for public prevention. Although the gold standard to identify the organism is culture and the methods are laborious and time-consuming. Since then, molecular-based approaches have been developed to detect DNA template of *B. anthracis* from various samples and widely applied as diagnostic tools. However, an efficient DNA extraction in adequate quantity and quality when using those molecular methods is an essential concern (Baunge *et al*., 2018). The route of infection for humans include cutaneous exposure, ingestion and inhalation. Humans with anthrax mostly harbor cutaneous form of the disease. (Castro and Okinaka, 2000; Kušar et al., 2012).

Bacillus anthracis is one of the major medically important species of *Bacillus* which has the characteristics of a non-motile, non-hemolytic which aids its virulence in establishing anthrax infection with a poly-D -glutamic acid capsule, the *Bacillus anthracis* resist phagocytosis by the tissue neutrophils and macrophage and the three (3) Bacillary proteins which are the lethal factor, the edema factor and protective antigen. *Bacillus anthracis* is sensitive to Penicillin, Tetracycline, Streptomycin antimicrobial agent. *Bacillus anthracis* is ubiquitous in nature, by existing as both free-living (nonparasitic) organism, and as parasitic pathogen to human. The infection of *Bacillus anthracis* can be transmitted to human through inhalation of the *Bacillus anthracis* spores, direct contact with the *Bacillus anthracis* spores and also through ingestion of the *Bacillus anthracis* spores in an under cooked meat with the infected *Bacillus anthracis* spores or on an infected vegetable. (CDC, 2018)

MATERIALS AND METHODS

The study area is Amassoma community under Southern Ijaw Local Government Area of Bayelsa State Nigeria. Bayelsa State is created out of the formal old River State in 1996. Bayelsa State is located within latitude 4°C - 15°C North and latitude 5°C - 23°C South. It is bounded by Delta State on the North, Rivers State on the East. The Population census of Bayelsa around 2006 was about 1.7 million people in which Amassoma community is one of the majorities.

SAMPLE COLLECTION

5g of non-sterile soil samples were collected randomly from different location in Niger Delta University Amassoma and community using a sterile universal container with the addition of sterile distilled water, then the samples where cultured in an aseptic condition at 37°C for 24 hours.

SAMPLE ANALYSIS

Isolation of the bacteria

From the 20 soil specimens collected, 5g was separated for identification process using Mannitol salt agar at mesophilic temperature for 24hours aseptically and sugar fermentation was carried out for Biochemical testing.

Identification of isolate

The different bacteria isolates were observed using colonial characterization of *Bacillus anthracis* which cannot utilize or ferment Mannitol as a sole source of sugar fermentation. The pH of the media retained it's alkaline state and separate colonies of the different isolate were subcultured using blood agar plate for beta and gamma hemolysis for *Bacillus cereus* and *Bacillus anthracis* respectively. Morphology of cells were then identified based on their Gram reaction as either Cocci, curve or straight Bacilli cells. (Blackburn *et al*., 2015)

Turbidity testing

Turbidity testing was carried out to differentiate *Bacillus anthracis* (negative) from *Bacillus cereus* (positive) by inoculating an isolate of the different plate into peptone broth and incubated at 37°C for 24 hours.

Susceptibility testing

A combination of both Gram positive and Gram-negative disk with different antibiotics were used to carry out the antimicrobial susceptibility testing using Mueller Hinton agar at 37°C for 24hours. The zone of inhibition was measured and compared to the standard chart accepted by CLSI. (Clinical Laboratory Standards Institute).

MOLECULAR ANALYSIS

DNA EXTRACTION (BOILING METHOD)

An overnight culture of pure isolate in nutrient broth was transferred into a 2ml Eppendorf tube and spun in a Denville 260D brushless micro centrifuge at 14000rpm for 4 minutes. The supernatant was decanted and 1000 micro liter of 0.5 of normal saline was added to the sediment and vortexed on Rltech XH-B vortex. The suspension was heated at 95^{O}c for 20mins and fast-

cooled for 10minutes. Thereafter, the suspension was spun again at 14000rpm for 3 minutes and 500 microlitre of the supernatant was extracted into 1.5ml of Eppendorf tube and stored in the freezer at -20°C for further downstream analysis.

DNA QUANTIFICATION

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrometer. The DNA concentrated was measured by clicking on the "measure" button. The DNA concentration was measured in ng/micro liter (5 - 100ng/micro liter) while purity level was determined by 260/280 absorbance (1.5 -2.0).

Amplification of 16S rRNA

The 16s rRNA of the rRNA gene of the Isolates were amplified using the 27F: 5'-AGAGTTTGATCATGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primer on a ABI 9700 applied biosystems thermal cycler at a final volume of 50 microlitre for 35 cycle. The PCR mix include: the 2X dream Taq master mix supplied by Inqaba, South African (tap polymerase, DNTPs, mgCl), the primer at a convention of 0.4M and the extracted DNA template. The PCR conditions were as Denaturation 950c for 5minutes, Annealing 520c for 30 seconds; Extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% Agarose gel at 120V for 15 minutes and visualized on a UV transilluminator. The amplicons were resolved on 1% agarose tinted with ethidium bromide and visualized using trans-illuminator.

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components includes 0.25ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10ul PCR primer, and 2 -10 ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

BLAST Analysis

The obtained sequences were edited using the bioinformatics algorithm trace edit and similar sequences were downloaded from Database. The sequences were aligned using clustalX. The

evolution history was inferred using the Neighbor joining method in MEGA6.0 (Saitou and Nei, 1987).

The bootstrap consequences tree inferred from 500 replicate (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes- Cantor method (Jukes and Cantor 1969)

RESULTS

Table 1: Isolates from the soil samples

Sample	*Bacillus species*	*Staphylococcus species*	*Mucor species*
1	-	-	+
2.	+	-	-
3.	-	+	-
4.	-	-	+
5.	-	+	-
6.	+	-	-
7.	-	+	-
8.	+	-	-
9.	-	+	-
10.	+	-	-
11.	+	-	-
12.	+	-	-
13.	+	-	-
14.	-	-	+
15.	-	+	-
16.	-	+	-
17.	-	+	-
18.	-	-	+
19.	-	-	+
20.	-	+	-

Table 2: Cultural characteristics and identification of *Bacillus species* isolate from Amassoma Niger Delta University soil sample

Sample code	Mannitol fermentation	Hemolysis	Gram staining	Catalase test	Turbidity
B1	NF	GH	GPB	+	-
B2	NF	BH	GPB	+	+
B3	NF	GH	GPB	+	-
B4	MF	BH	GPB	+	+
B5	MF	BH	GPB	+	+
B6	MF	GH	GPB	+	-
B7	MF	BH	GPB	+	+

Key: B= Bacillus, MF= Mannitol fermenter, NF = non fermenter, GH = gamma hemolysis, BH = beta hemolysis, + = positive, - = negative and GPB = Gram positive Bacilli.

B1— College of Health Science dumpsite soil, B2---Main campus pavilion B3—Main Campus statutes park soil, B4--- Chaplite environment soil, B5—Main Campus cow dump. B6—College of Health Science car park soil B7: Main Campus animal farm soil samples.

Table 3: Percentage distribution of Susceptibility testing of *Bacillus species* isolate from Niger Delta University soil sample.

Sample Code	CPX S(%)	SXT R(%)	S S(%)	PN R(%)	CEP R(%)	OFX S(%)	NA R(%)	CN S(%)	AU R(%)

B1	30(17.14)	14(13.59)	20(12.42)	11(12.36)	13(13.68)	25(14.97)	14(14.89)	15(16.85)	13(13.68)
B2	21(12.0)	12(11.65)	20(12.42)	14(15.36)	13(13.68)	25(14.97)	12(12.77)	18(13.25)	14(15.73)
B3	30(17.14)	15(14.56)	30(18.63)	13(14.48)	14(14.74)	27(16.17)	12(12.77)	16(14.57)	12(14.44)
B4	30(17.14)	15(14.56)	34(12.12)	15(16.85)	13(13.68)	30(17.96)	15(16.96)	15(16.85)	13(13.68)
B5	21(12.00)	14(13.59)	20(12.42)	14(15.73)	13(13.68)	20(11.98)	14(14.89)	18(13.25)	14(15.73)
B6	22(12.57)	13(12.62)	24(14.91)	14(15.73)	10(10.53)	20(11.98)	14(14.89)	16(14.57)	12(14.44)
B7	21(12.00)	20(19.42)	13(8.07)	21(26.60)	19(2.00)	20(11.98)	15(16.96)	16(14.55)	20(19.42)

Key: CPX=Ciprofloxacin, SXT= Septrin, S= Streptomycin, PN= Ampicillin, CEP= Ceporex, OFX= Ofloxacin NA= Nalidixic acid, PEF= Reflacine, CN= Gentamicin and AU= Augmentin S = Sensitive and R = Resistance**.**

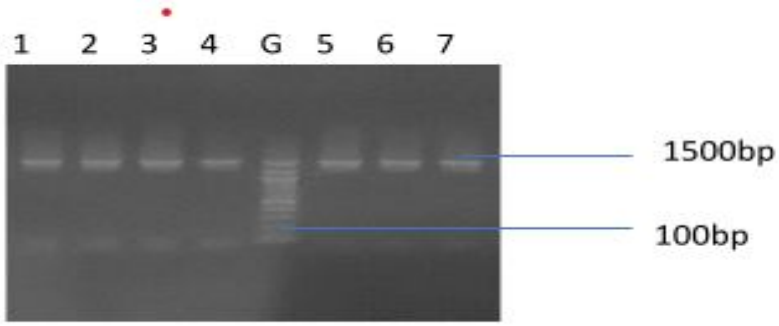

PLATE: Agarose gel electrophoresis of some selected bacterial isolates. Lanes 1 – 7 represent 16SrRNA gene bands (1500bp). Lane G represents the 100bp Molecular ladder.

Plate 1: Agarose gel electrophoresis of some selected bacterials isolates. Lanes 1- 7 represent 16SrRNA gene bands (1500bp). Lane G representsthe 100bp molecular ladder.

Table 4: BLAST Analysis of *Bacillus species* isolate from Amassoma Niger Delta University soil sample.

Sample code	Phenotypic	Genotypic
B1	*Bacillus species*	*Lysinibacillus macrolides*
B2	*Bacillus species*	*Bacillus cereus*
B3	*Bacillus species*	*Lysinibacillus macrolides*
B4	*Bacillus species*	*Bacillus subtilis*
B5	*Bacillus species*	*Bacillus subtitis*
B6	*Bacillus species*	*Lysinibacillus capsici*
B7	*Bacillus species*	*Bacillus subtitis*

DISCUSSION

Although Culture has been the gold standard for diagnosis. Identification of the agents by routine bacteriological methods is of utmost importance. *Staphylococcus*, *Mucor* and *Bacillus* species were isolated from each soil sample in partially agreement with a study by (Prashanti *et al.,* 2021) who found other communities of Bacteria in soil and disagreed with a study conducted by (Salam *et al*., 2018) by which antimicrobial activity was against *Bacillus.*

Also B2, B4, B5 and B7 were indicative of beta hemolysis on blood agar which is in line with (Blackburn *et al*., 2015) as a growth characteristic of *Bacillus cereus* on blood agar plate with a wide clearing yellow coloration while B1, B3 and B6 were indicative of gamma hemolysis in contrast to the study by Klincho *et al*., 2003 who got beta hemolysis for *Bacillus anthracis*. The bacteria isolate from B1 to B7 were Gram positive Bacilli in chains and were catalase positive with the release of an active bubble formation with hydrogen peroxide due to the presence of catalase enzyme as also reported by Babiker *et al*., 2016. Also, sample codes B1, B3, and B6 were negative to turbidity testing according to (CDC, 2020) which was characterized by *Bacillus anthracis* while the rest were positive to turbidity testing for *Bacillus cereus* and other related

Bacillus species. Phenotypic tests are essential for *B. anthracis* identification. However, classic phenotypic characteristic may not be present in all B. anthracis strains. Antimicrobial susceptibility testing showed high resistance to the common antibiotics available contributing to a a threat to public health.

16S ribosomal RNA (rRNA) gene sequencing is a popular alternative to traditional methods and provides several advantages (Salipante *et a*l., 2013). Sequencing results showed an advantage of genotypic features above phenotypic features in conjuction with a study by Kovac *et al*., 2016. *Bacillus anthracis* exists in the soil as spores contributes to environmental health hazard for its ability to survive for a longer period and can also be used as biothreat (Goal, 2015).

Anthrax infection could be by inhalation of spores, or zoonotic (from infected animals), Staff, students which were always getting in contact to the sites should take preventive measures like personal hygiene.

Conclusion

Bacillus anthracis infection is one of those neglected tropical diseases. The micro-organisms are all around us, therefore more researches should be conducted for isolation and identification of these soil microorganisms genotypically, improper identification of the etiological agent may lead to myriad of problems.

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