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Distinctive Molecular typing of 16S rRNA of Bacillus species isolated from farm settlement.

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ABSTRACT

Introduction: There are numerous methods of isolating and detecting organisms that are similar and closely related; one of the most reliable method is molecular typing of 16S rRNA. Apart from being omnipresent as a multigene family, or operons; it is evolutionarily stable; the 16S rRNA gene (1,500 bp) is large enough for informatics purposes.

Materials and Method: This study employed molecular sequencing of 16S rRNA by Sanger method to reveal the specific organisms' nucleotides and blasting (BLASTn) to show the similarities between the resulting organisms and existing organisms. The 16S rRNA remains the best choice of identification process for bacteria because of its distinguishing sizes and evolutionary stability.

Results: All isolates were Gram positive rods and were positive in Biochemical tests such as oxidase, catalase, citrate, and protease but were in turn negative in coagulase and indole test tests. On sensitivity test; 80% of all the isolates were resistant to common antibiotics except ciprofloxacin and ceftriaxone. Based on the sequence difference in the variable region (V1) of 16S rRNA as observed from the molecular sequencing results; four isolates out of ten were identified. Six were different strains of *B cereus*. Others isolates include: *wiedmannii, thuringensis, toyonensis* and *pseudomycoides*. Sequence analysis of the primer annealing sites showed that there is no clear-cut difference in the conserved region of 16S rRNA, and in the *gyrB* gene, between *B. cereus* and *B. thuringiensis* strains. Phylogenetic analysis showed that four isolates showed high similarity to each other; hence the limited number of deletions when subjected to alignments by maximum neighborhood joining parsimony using MEGA X software. *B. toyonensis, B. wiedmannii* and *thuringensis* were distantly related

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Introduction

Authors Pathogens cause illness and death in some countries and it also causes infections and gastrointestinal diseases in other countries thereby causing public health concern. Pathogens are organisms capable of causing diseases. Reliable methods are needed for the detection of pathogens due to pathogen evolution as a result of new human habits or new industrial practices.

Microbial classification of organisms ranges from genus to specie level depending upon the technique used either phenotypic or genotypic. Presently, molecular methods now obtain advances to allow utilization in microbiology [1]. There are numerous molecular methods which are of fast and simple application to the detection of pathogen. Among the pathogens involved in human health, *Bacillus cereus* is interesting due to their ability to survive in various habitats [2].

The genus *Bacillus* is aerobic or facultative anaerobic bacteria, gram positive spore forming rod shaped bacteria. Which can be characterized by two morphological forms, the vegetative cell which range from 1.02 to 1.2 um in width and from 3.0 to 5.0 in length, it can be straight or slightly curve, motile or non-motile, and the endospore (the non-swelling sporangium). The genus *Bacillus* is been characterized by the presence of endospore, which is not more than one per cell and they are resistant to many adverse environmental conditions such as heat, radiation, cold and disinfectants. It can also respire either in the presence or absence of oxygen [3]. Cell diameter of *Bacillus cereus*, sporangium and catalase test do not allow differentiation, whereas important in differentiation among *B. anthracis, B. cereus, B. thuringiensis* can be considered by parasporal crystals and the presence of capsule. [4] Showed a *B. thuringiensis* strain capable of producing a capsule resembling that of *B. anthracis*. Most species of the genus display a great kind in physiological characteristics such as degradation of cellulose, starch, pectin, agar, hydrocarbons, production of enzymes and antibiotics and other characteristic such as acidophile, alkalinophile, psychrophile, and thermophile's which allows them to adapt to various environmental conditions [5]. In differentiating between species of the genus Bacillus it was difficult at early attempts when endospore formation and aerobic respiration were the main character used for classification. As reported by many authors that at molecular method level, the differentiation between *B. thuringiensis* and *B. cereus* is also very difficult.

B. cereus can survive at the temperature between 4°c and 55°c. The mesophile strains can grow between the temperature of 10°c and 42°c, while psychotropic strains can survive at 4°c, whereas other strains are able to grow at 52 to 55°c. *B. cereus* vegetative cells grow at pH between 1.0 and 5.2. Heat resistant strain can survive and multiply in wet low acid foods in temperature ranging from 5 to 52°c. The survivability of *B. cereus* spores at 95°c decreases when the pH level decreases from 6.2 to 4.7 [6]. *B. cereus* can grow in the presence of salt with concentration up to 7.5% depending on the pH value.

B. thuringiensis possesses a protein crystal that is toxic to insects. This toxin protein was first known as parasporal crystalline inclusion but was later referred to as π - endotoxin or in other ways known as insecticidal crystal protein [7]. Strains of *B. thuringiensis* bacteria possess a wide range of specificity in various orders of insects such as Lepidoptera, dipteral, coleoptera. These strains of bacteria produce crystalline proteins known as cry protein during sporulation. When B. thuringiensis infects an insects, it will cause the insect to loose appetite, enhances slow movement and over time the insect will die due to crystals of proteins that have been dissolved in the insect's stomach.

In the cultivation of vegetable crops, the plant can be attack by many types of pests. Hence, in overcoming pest attacks farmers often use pesticides that contain active synthetic materials. Many negative effects arise from the folly use of chemical pesticides. Among the negative effect is the increase of pest population, resistance, death of natural enemy population and increase in residue level on Agricultural product which makes it unsafe for public consumption [8]. Therefore, it is necessary to find an alternative method in the control of crop pest. The best alternative that can be done is to replace the chemical insecticide with biological control which involves the use of living things in the form of microorganisms. In these profiling microbial communities, the main objective is to identify which bacteria and how much they are present in the environments. Most microbial profiling methods focus on the identification and quantification of bacteria with already sequenced genomes. Further, most methods utilize information obtained from entire genomes. Homology-based methods such as [1–4] classify sequences by detecting homology in reads belonging to either an entire genome or only a small set of marker genes. Composition-based methods generally use conserved compositional features of genomes for classification and as such they utilize less computational resources.Using the 16S rRNA gene instead of whole genome information is not only computational efficient but also economical; Illumina indicated that targeted sequencing of a focused region of interest reduces sequencing costs and enables deep sequencing, compared to whole-genome sequencing. On the other hand, as observed by [8], by focusing exclusively on one gene, one might lose essential information for advanced analyses. We, however, will provide an analysis that demonstrates that at least in the context of oral microbial communities, the 16S rRNA gene retains sufficient information to allow us detect unknown bacteria [9, 10]. This study aimed at employing 16S rRNA as an instrument of identification of seemingly close *Bacillus species.*

Abbreviations

BLAST, Basic Local Alignment sequence Tools; PCR, Polymerase Chains reactions; rRNA, ribosomal RNA;

Material and methods

T Sample collection. Soil samples were collected from three sources from Rice, Sugar Cane, vegetables and abandoned farmland in January 2019. The samples were labeled serially from Sample 1 to Sample 10 (S_1 to S_{10}).

Bacterial culture: A serial dilution of 10 folds was performed. Bacterial suspension was diluted (10^{-10}) with saline water and 100 µl of bacterial suspension werespread on Nutrient Agar plate and incubated for 24 hours. Bacterial colonies were isolated and grown in Nutrient Broth and nutrient agar. Other microbiological solid agar used include: Chocolate, Blood Agar, EMB, MacConkey, Simon citrate, MRS Agar. Bacteria were characterized by conventional technique by the use of

morphological appearance and performance on biochemical analysis [11].

Identification of bacteria: The identification of bacteria was based on morphological characteristics and biochemical tests carried out on the isolates. Morphological characteristics observed for each bacteria colony after 24 h of growth included colony appearance; cell shape, color, optical characteristics, consistency, colonial appearance and pigmentation. Biochemical characterizations were performed according to the method of [12]

Catalase test: A small quantity of 24 h old culture was transferred into a drop of 3% Hydrogen peroxide solution on a clean slide with the aid of sterile inoculating loop. Gas seen as white froth indicates the presence of catalase enzyme [13] on the isolates.

DNA Extraction Processes

The extraction processes was in four phase which are:

Collection of cell, lyses of cell, Collection of DNA by phenol, Concentration and purification of DNA.

Collection of cell: the pure colony of the bacteria culture was inoculated into a prepared sterile nutrient broth. After growth is confirmed by the turbidity of the culture, 1.5ml of the culture was taken into a centrifuge tube and was centrifuge at 5000 rpm for 5 minutes; the supernatant layer was discarded leaving the sediment.

Lyses of cell: 400 microns of lyses buffer is added to the sediment and was mixed thoroughly and allow to stand for five minutes at room temperature (25°c). 200 microns of Sodium Dodecyl Sulfate (SDS) solution was added for protein lyses and was mixed gently and incubated at 65°c for 10 minutes.

Collection of DNA by phenol; 500 microns of phenol chloroform was added to the solution for the separation of DNA, it was mixed completely and centrifuge at 10,000 rpm for 10 minutes. The white pallet seen at the top of the tube after centrifugation is separated into another sterile tube and 1micron of Isopropanol is added and incubated for 1hour at -20°c for precipitation of DNA. The DNA is seen as a colorless liquid in the solution.

Concentration and purification of DNA: the solution was centrifuge at 10,000 rpm for 10 minutes. The supernatant layer was discarded and the remaining DNA pellets was washed with 1micron of 17% ethanol, mixed and centrifuge at 10,000 rpm for 10 minutes. The supernatant layer was discarded and air dried. 60 micron TE. Buffer was added for further dissolving of the DNA which was later stored at -40°c until it was required for use [14].

PCR Amplification

This requires the use of primers (Forward and Reverse), polymerase enzyme, a template DNA and the d pieces which includedddATP, ddGTP and ddTTP, ddNTP. All this are called the master mix.

The PCR reactions consist of three main cycles.

The DNA sample was heated at 94^0c to separate the two template of the DNA strand which was bonded by a hydrogen bond. Once both strand are separated the temperature is reduced to 57^0c (Annealing temperature). This temperature allows the binding of the forward and reverse primers to the template DNA. After binding the temperature is raised back to 72^0c which leads to the activation of polymerase enzyme and its start adding d NTPs to the DNA leading to the synthesize of new strands. The cycles were repeated several times in order to obtain millions of the copies of the target DNA [15].

Preparation of Agarose Gel

One gram (1 g) of agarose for DNA was measured or 2 g of agarose powdered will be measured for PCR analysis. This done by mixing the agarose powder with 100 ml 1×TAE in a microwaveable flask and microwaved for 1-3 minutes until the agarose is completely dissolved (do not over boil the solution as some of the buffer will evaporate) and thus alter the final percentage of the agarose in the gel. Allow the agarose solution to cool down to about 50°c then after five minutes 10µL was added to EZ vision DNA stain. EZ vision binds to the DNA and allows one to easily visualize the DNA under ultra violet (UV) light. The agarose was poured into the gel tray with the well comb firmly in place and this was placed in newly poured gel at 4°c for 10-15 mins or it sit at room temperature for 20-30 mins, until it has completely solidified[16].

Loading and Running of samples on Agarose gel

The agarose gel was placed into the chamber, and the process of electrophoresis commenced with running buffer introduced into the reservoir at the end of the chamber until it the buffer covered at least 2millimeter of the gel. It is advisable to place samples to be loaded in the correct order according to the lanes they are assigned to be running. When loading the samples keep the pipette tip perpendicular to the row of the wells as by supporting your accustomed hand with the second hand; this will reduce the risk of accidentally puncturing the wells with the tip. Lower the tip of the pipette until it breaks the surface of the buffer and is located just above the well. Once all the samples have been loaded it is advised to always avoid any movement of the gel chamber. This might result in the sample spilling into adjacent well. Place the lid on the gel chamber with the terminal correctly positioned to the matching electrodes on the gel chamber black to black and red to red. Remember that DNA is negatively charged hence the movement of the electric current from negatively charged to the positively charged depending on the bandwidth in Kilobytes. Once the electrode is connected to the power supply, switch ON the power supply then set the correct constant voltage (100) and stopwatch for proper time. Press the start button to begin the flow of current that will separate the DNA fragment. After few minutes the samples begins to migrate from the wells into the gel. As the DNA runs, the diaphragm moves from the negative electrode towards the positive electrode [17].

PCR mix Components and Sanger Sequencing

This is made up of primers which is both Forward and Reverse, the polymerase enzyme (Taq), a template DNA and the pieces of nucleotides which include: ddNTP, ddATP, ddGTP and ddTTP. Note that the specific Primer's sequences for bacterial identification is: 785F 5' (GGA TTA GAT ACC CTG GTA) 3', 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3', 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3', 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' in Sanger Sequencing techniques.

BLAST

The resulting genomic sequence were assembled and submitted in GenBank at NCBI for assignment of accession numbers. The resultant assertion numbers were subjected to homology search by using Basic Local Alignment Search Tool (BLAST) as NCBI with the assertion number MW362290, MW362291, MW362292, MW362293, MW362294 and MW362295 respectively. Whereas, the other isolates' accession numbers were retrieved from NCBI GenBank which are:AB 738796.1, JH792136.1, MW 015768.1 and MG745385.1.MEGA 5.2 software was used for the construction of phylogenetic tree and phylogenetic analysis.

Table 1: Morphological Description of Growth of the bacteria on a variety of culture media

Media types	Colony characteristics
EMB agar and MacConkey agar	No growth
Chocolate agar containing 5% fetal calf serum	Grayish white, round, opaque, thick ridges, smooth, moist, medium-sized colony
MRS agar	Pure white, mucoid round, opaque, drying, medium-size colony
Rabbit blood agar	Gray white, round, completely hemolytic, opaque, flat, drying, medium-size colony
Nutrient agar	Grayish white, opaque, flat, parched, medium-size colony

All the organisms possess 100% identities, 0% gaps and 0.0% E. value which indicated that the organisms are closely related to the existing organisms. The use of 16S rRNA is the best identification process for bacteria because 16S rRNA gene has a distinguishing size of about 500 bases until 1500bp. Rather than using 23S rRNA which is of higher variation, The 16S rRNA is adopted in prokaryotes. 18S rRNA is used for identification in Eukaryotes

Results

The results of both the conventional morphological and cultural identification was correlated with the molecular sequencing results. Six isolates were confirmed *B. cereus* species while the other four isolates were. *B. wiedmannii, B. thuringiensis, B. toyonensis* and *B. pseudomycoides.*The 16S rRNA sequence of six isolates MW 362290.1- MW362295.1 were assigned accession numbers and deposited in the GenBank while the other four sequences were aligned to those available in the NCBI database. The alignment results showed closely relatedness to LT844650.1with an identity of 100% to 92.2% as above. The six isolates of *Bacillus cereus* great evolutionary relatedness as shown in the phylogenetic tree constructed using MEGA X software.

Figure 1

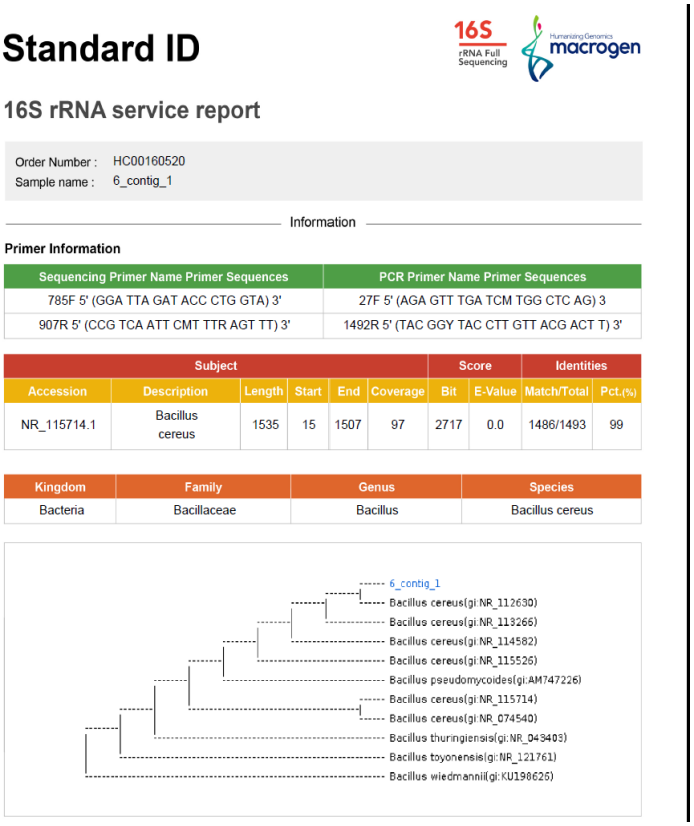

Table 2. Summary closely related nucleotides compared with NCBI GenBank

Sample	Query I.D.	Subject I.D.	Query length	Subject length	Identities	E.value	Gaps
S_1	MW362290.1	MT538265.1	1493	1535	100%	0.0	0/1493(0%)
S_2	MW362291.1	MT538265.1	1493	1535	100%	0.0	0/1493(0%)
S_3	MW362292.1	MT052668.1	1493	1540	100%	0.0	0/1493(0%)
S4	MW362293.1	MT538265.1	1493	1535	100%	0.0	0/1493(0%)
S_5	MW362294.1	MT538265.1	1493	1535	100%	0.0	0/1493(0%)
S_6	MW362295.1	MT292101.1	1543	1539	100%	0.0	0/1483(0%)
S_7	LT844650.1	AB 738796.1	1748	1492	92%	0.0	53/1555 (7%)
S_8	JH792136.1	LC 602955.1	1465	1462	92%	0.0	53/1555 (7%)
S_9	LT844650.1	MW 015768.1	1425	1425	100%	0.0	0/1481 (0%)
S_{10}	LT844650.1	MG745385.1	1481	1481	100%	0.0	0/1481 (0%)

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Discussion

The results obtained in this study is consistent with the previous studies in other countries22,23 The results of the phylogenetic analysis of the 16S rRNA isolate of in this study was similar to the housekeeping genes proposed by [18, 19]. In comparing this study with the earlier study, B. cereus group comprising other species of Bacillus was hypothesized to be considered to form a single species with different ecotypes and pathotype. This study was able to phenotypically differentiated *B. thuringiensis, B. pseudomycoides, B. toyonensis, B. wiedmannii* and *B. cereus sensu strito*. Despite differences at the colonial appearance level, the 16S rRNA sequences have homology ranging from 100% to 92% providing insufficient resolution at the species level [6, 7, 18].After analysis through various methods, the strain was identified as Gram-positive bacteria of *Bacillus cereus* with a homology of 99.4%. Cohan [20] demonstrated that 95–99% of the similarity of 16S rRNA gene sequence between two bacteria hints towards a similar species while >99% indicates the same bacteria.The phylogenetic tree showed that *B. toyonensis, B. thuringiensis* and *B. wiedmanni*are the outgroups of B. cereus group while *B. pseudomycoides* are most closely related to *B. cereus* group [19, 21, 22].

Conclusion

In the area of molecular epidemiology, genotypic typing method has greatly increased our ability to differentiate between micro-organisms at the intra and interspecies levels and have become an essential and powerful tool. Phenotypic method will still remain important in diagnostic microbiology and genotypic method will become increasingly popular.

After analysis through various methods, the strain was identified as Gram-positive bacteria of *Bacillus cereus* with a homology of between 100% and 92.3%.

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Collate acknowledgments in a separate section at the end of the article before the references, not as a footnote to the title. Use the unnumbered Acknowledgements Head style for the Acknowledgments heading. List here those individuals who provided help during the research.

Conflicts of interest

The Authors declare that there is no conflict of interest.

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