International Journal of Biochemistry, Bioinformatics and Biotechnology Studies
Vol.3, No.1, pp.21-30, February 2018
Published by European Centre for Research Training and Development UK (www.eajournals.org)

CLASSICAL IDENTIFICATION, 16S RDNA SEQUENCING, AND MOLECULAR CHARACTERIZATION OF *BACILLUS* SPECIES FROM CONVENIENCE FOODS

Aruw C. E.[1] And Ogunlade S. T.[2]

[1]Department of Microbiology, Federal University of Technology, P.M.B. 704, Akure, Nigeria
[2]Department of Mathematical Sciences, Federal University of Technology, P.M.B. 704, Akure, Nigeria

ABSTRACT: *Identification of microorganisms is central to the study of microbiology at all levels of research. The methods employed are also important. It is however very pertinent that scientists the world over continuously improve on the method of microbial identification for greater efficiency. A study was conducted to isolate and identify Bacillus species from some ready-to-eat (RTE)/convenience food samples. The Bacillus species isolated were identified by using the classical method. The same isolates were further identified via use of the 16S rDNA sequencing method. The classical method identified all bacilli isolates as members of a precise species in the genus Bacillus, but with discrepancies observed in 3 out of 9 identified cases (33.3%) when comparison was made with PCR/sequencing method. PCR/sequencing method provided results which were in accordance with both classical and genotypical identification in more than 70% of cases. This study emphasized the presumptive nature of classical methods in identifying Bacillus species/strains, without additional sensitive and molecular methods. Identities from the PCR method hold greatest sway and are regarded as most reliable as it involves the analysis of genetic sequences of this group of microorganisms.*

KEYWORDS: Classical, Identification, Bacillus, Convenience Foods, PCR

INTRODUCTION

The genus *Bacillus* currently comprises in excess of 60 species, commonly found in the environment and as laboratory contaminants. *Bacillus* species are Gram positive rods often arranged in pairs or chains with rounded or square ends and usually have a single endospore. The endospores are generally oval and are very resistant to adverse conditions. Sporulation is not repressed by exposure to air. Many *Bacillus* species are haemolytic, a useful characteristic in differentiating them from *B. anthracis* (which is non-haemolytic). They are aerobic or facultatively anaerobic and most species are motile (a notable exception is *Bacillus anthracis*) by peritrichous flagella. Most species are oxidase positive, which may lead to confusion with *Pseudomonas* species, especially if the *Bacillus* species are poorly stained. They are usually catalase positive and metabolise carbohydrates by fermentation. Significant isolates should usually be referred to a Reference Laboratory for confirmation of identity and toxin testing (PHE, 2014).

Dairy products, fatty foods, bread, cakes and pastries, seafood can easily be contaminated with *Bacillus* spp. *Bacillus cereus* (*B. cereus*) can cause food poisoning resulting in gastroenteritis. *Bacillus* species are ubiquitous in nature and can be found in soil or in a variety of dried foods such as grains, legumes, starches and spices as vegetative cells and endospores (Rusul and Yaacob, 1995). According to Food and Drug Administration of the United Stated, food poisonings due to *B. cereus* group have two different clinical syndromes, diarrheal and emetic (vomiting) syndrome. The emetic type causes vomiting after 0.5–6 h of ingestion (Ehling-

Print ISSN: 2397-7728, Online ISSN: 2397-7736

Schulz *et al*., 2005) and diarrhoeal type causes abdominal pain and diarrhoea after 8 to 16 h of consumption. The diarrheal syndrome has been associated with a wide variety of food including meats, milk, vegetables and fish. The emetic syndrome has been generally associated with rice products, starchy foods such as potato, pasta, noodles, spaghetti, pastry and cheese products (Shinagawa, 1993; Granum and Lund, 1997).

Based on the report of European Food Safety Agency (2005), 1–33% of food-borne poisonings are caused by *B. cereus*. Food poisoning is caused with presence of bacteria in food due to improper food preparation or cooking process and exposure of food to temperatures of 30ºC. Common food poisonings are usually mild, but deaths due to food poisoning are also reported. Food poisoning occurs within 48 hours after consumption of contaminated food or drink. The symptoms include nausea, vomiting, diarrhoea and abdominal pain. Most cases of food poisoning are caused by bacteria, viruses or toxins and chemicals (Drobniewski, 1993). Some of *B. cereus* outbreaks are under reported as the illness associated with these bacteria limit itself and does not become severe. A recent survey on culture practices for outbreaks of apparent food borne illness showed that 20% of state public health laboratories do not make *B. cereus* testing routinely available. The survey also found that most of food handlers (in food stalls and restaurants) were unaware that cooked rice was a potentially hazardous food (Todar, 2009).

Gram positive and aerobic spore-forming bacilli belonging to the genus *Bacillus*, and other related species play important roles in food poisoning and spoilage. There is however some difficulty due to the lack of standard methods for identification of members of *Bacillus* species in food testing laboratories (Mugg *et al*., 2013). Species differentiation of the genus is complex, and in some instances in routine laboratories, a combination of Gram stain and colonial appearance may be regarded as sufficient indication of a *Bacillus* species being present in a clinical sample. Although the use of morphological and physiological tests have provided the best means available for laboratories to identify organisms, these methods have proven to be quite laborious, inconsistent and generally unreliable for this group of microorganisms (ibid). As a result of the absence of reliable and standardized methods for the identification of this group of organisms, investigators have generally focused on the isolation and identification of *Bacillus cereus* as a causative agent of food related illnesses. However, numerous other investigations have demonstrated that a considerably larger range of species can cause food related illness (Kramer and Gilbert, 1989; Gilbert *et al*., 1981).

Prevailing neglect of *Bacillus* identification may be attributed to two factors. Firstly, the diagnostic tests used; many of the classical tests for *Bacillus* described by Gordon *et al.* 1973 require special, selective/differential media. These are very time consuming and expensive to prepare. Many of these media have short shelf lives resulting in considerable wastage if their use is infrequent. The requirement for media containing unusual ingredients increases the familiar problems of test standardization (Sneath and Collins, 1974); and inconsistent results may be obtained in consequence. Any new scheme for *Bacillus* identification should therefore use widely available and standardized materials for performing a good number of rapid tests which give reproducible results. The second factor leading to neglect of *Bacillus* identification is the character of the genus. *Bacillus* is an unusually wide taxon which contains most aerobic endospores-forming rods. In terms of DNA base ratios it is the equivalent of some bacterial families (Priest, 1981). Furthermore, some species are ill-defined, existing with closely related species as complexes or in which the boundary of a particular species is difficult or impossible to identify. Even in well established species there is considerable variation between strains. Thus, classical test schemes using few characters often do not permit identification of atypical and intermediate strains and in spite of the excellent work of Gordon and her colleagues in

Print ISSN: 2397-7728, Online ISSN: 2397-7736

1973, as well as others, it is widely agreed that there is considerable room for improvement in the taxonomy of the genus and that a study of new isolates, particularly, is important.

There has been very little study on the isolation and molecular characterization of *B. cereus* and other *Bacillus* species in ready-to-eat rice products and pastries in Nigeria. The aim of this study was to isolate and carry out classical identification and molecular characterization of *B. cereus* and other *Bacillus* species from some ready-to-eat food samples. The results from both methods would provide for good comparison. This paper also aimed at providing initial data which would encourage the use of molecular identification methods in the assay for *Bacillus* species especially in developing countries. It is also the hope that this class of microorganisms would be included in routine food test for RTEs.

MATERIALS AND METHODOLOGY

Sample collection was carried out according to the methods of Cheesbrough (2006) and Fawole and Oso (2001). A total of sixty RTE food samples (10 samples each of 3 different types of pastry products – Egg roll, Meat pie, and Buns; 10 each of different rice products – White rice, Jollof rice, and Fried rice) were purchased from different food vending sites and cafeterias within a period of ten weeks. Food samples purchased were appropriately labelled and transferred to the laboratory for immediate analysis.

Growth and Isolation of Bacterial Cultures

Bacillus bacteria were isolated from collected convenience food samples using the serial dilution technique with pour plating unto Nutrient agar (NA); and also with spread plating unto HiCrome *Bacillus* agar (HiMedia), a selective/differential isolation media, for assessment of *Bacillus* species. The fourth dilution was used for plating unto these media. Culture media were prepared according to manufacturer's specification and sterilization of materials was done in an autoclave at 121^{o}C for 15 minutes.

Preservation of bacteria isolates

Discrete colonies isolated and purified by repeated sub-culturing were preserved according to Olutiola *et al*. (1991), on slants at 4^{o}C for further characterization.

Classical Identification

Morphological and biochemical tests to identify isolates were carried out using the methods of Fawole and Oso (2001) and Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986). Biochemical tests carried out in the conventional method include fermentation of carbohydrate, catalase, motility, coagulase.

Molecular characterization of Bacilli isolates

Isolation of genomic DNA from bacteria

DNA was extracted from 1 ml of bacterial culture. The culture was pelleted by centrifuging at 12,000rpm for 5 min. Pellets were then treated with lysis buffer and protease enzyme and incubated at 65^{o}C for 1 hr. Nucleic acids were precipitated with isopropanol by centrifuging at 10,000rpm for 10 min, washed with 1 ml of 70% ethanol solution and dissolved in 0.1 ml of TE buffer. The purity and quantity of DNA were examined by recording its UV absorption

Print ISSN: 2397-7728, Online ISSN: 2397-7736

spectrum and running on 1% agarose gel electrophoresis.

Sequence determination of 16S rDNA

The DNA isolated was amplified using 16S rDNA universal primers and sequenced for the identification of *Bacillus* species at molecular level. Amplification of the PCR products of expected size was confirmed by electrophoresis. The sequence of the 16S rDNA was determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer. The gene sequences obtained in this study were compared with known 16S rDNA gene sequences in the National Centre for Biotechnology Information (NCBI) GenBank database.

RESULTS

Plate 1 shows the electrophorogram from separated and amplified DNA bands purified and amplified DNA extracts. Table 1 shows the bacilli organisms identified using the classical method of identification. A comparison of isolates identified by this method and the PCR/sequencing method is also depicted. Only the Ribos C microorganism was not recognised as a member of the *Bacillus* genus via the PCR method. Figures 1-8 show relationship between the different species of the *Bacillus* genus using the BioPython programming language. Figure 7 showed the closest relationship between the genomic relations while Figure 2 showed the least relationship. Figures 9 showed the genetic relatedness of *Bacillus* species isolated from ready-to-eat (RTE) foods, while figure 10 better depicted the phyletic relationships between isolated *Bacillus* species and NCBI database closely related sequences.

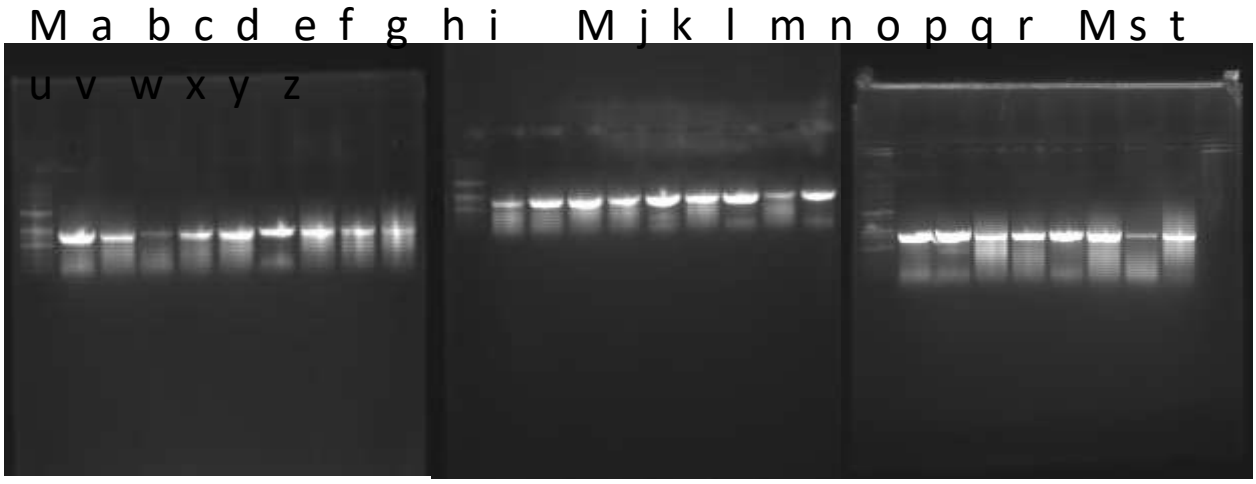

Plate 1: Separated and amplified DNA bands from gel electrophoresis

Table 1: Identification of *Bacillus* species by classical method and 16S rDNA sequencing

Organism	Sample source	Identity by Classical	Identity by 16S sequencing
Ribos B	Buns	*Bacillus cereus*	*Bacillus licheniformis*
Ribos C	White rice	*Bacillus subtilis*	*Comamonas* sp.

Print ISSN: 2397-7728, Online ISSN: 2397-7736

Ribos D	Egg roll	*Bacillus sphaericus*	*Bacillus atrophaeus*
Ribos G	Meat pie	*Bacillus licheniformis*	*Bacillus thuringiensis*
Ribos H	Egg roll	*Bacillus subtilis*	*Bacillus amyloliquefaciens*
Ribos J	Fried rice	*Bacillus subtilis*	*Bacillus safensis*
Ribos L	Meat pie	*Bacillus amyloliquefaciens*	*Bacillus subtilis*
Ribos M	Buns	*Bacillus mycoides*	Uncultured *Bacillus* sp. clone
Ribos S	Jollof rice	*Bacillus cereus*	*Bacillus cereus*

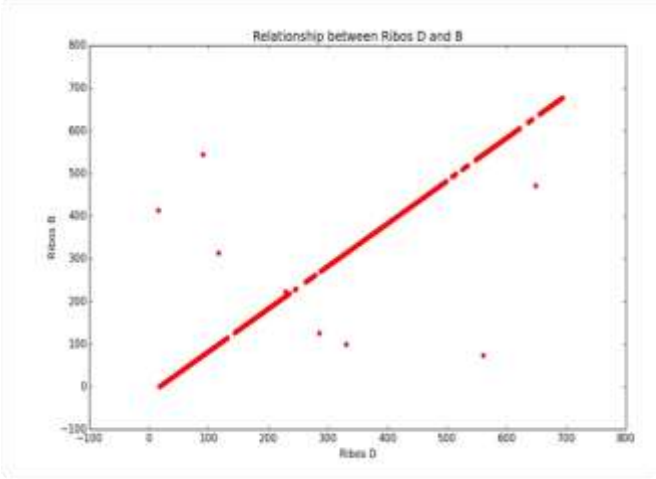

Fig. 1: DNA sequence relations between ribos D and B isolates

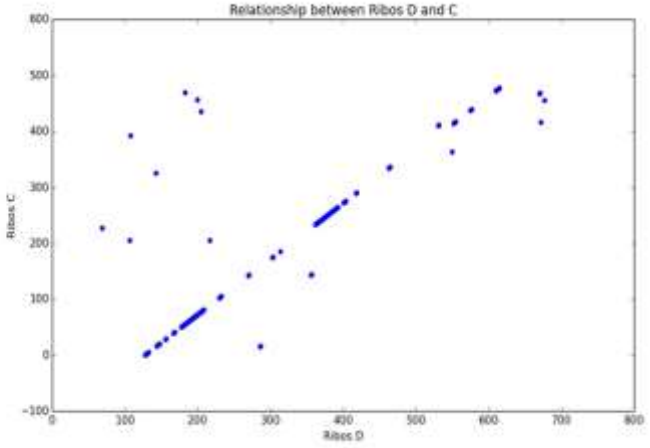

Fig. 2: DNA sequence relations between ribos D and C isolates

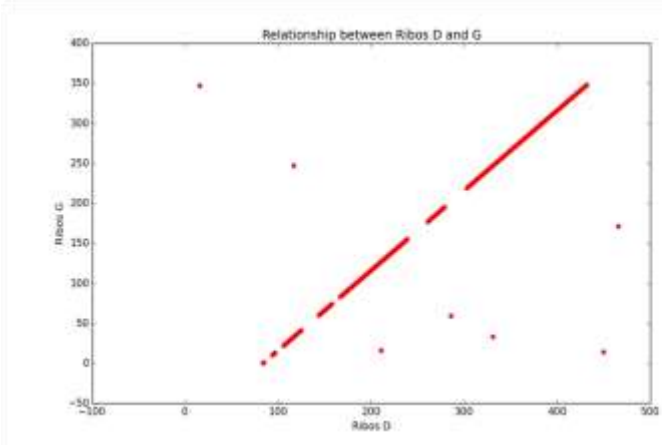

Fig. 3: DNA sequence relations between ribos D and G isolates

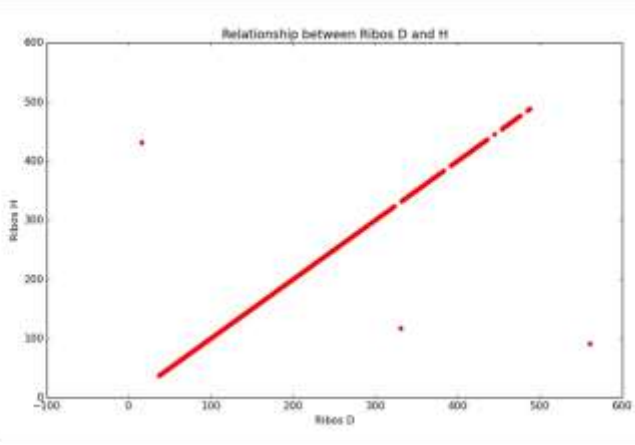

Fig. 4: DNA sequence relations between ribos D and H isolates

Print ISSN: 2397-7728, Online ISSN: 2397-7736

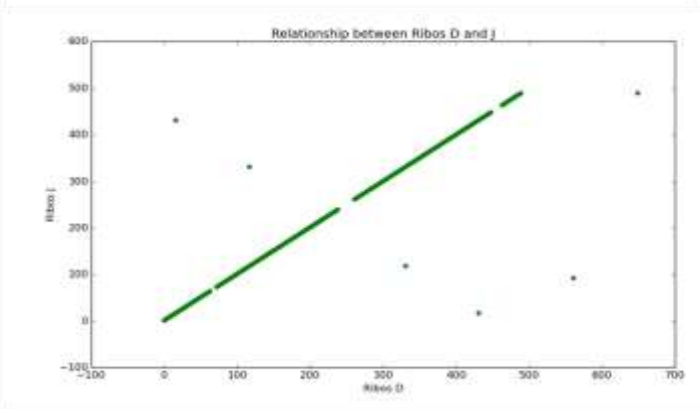

Fig. 5: DNA sequence relations between ribos D and J isolates

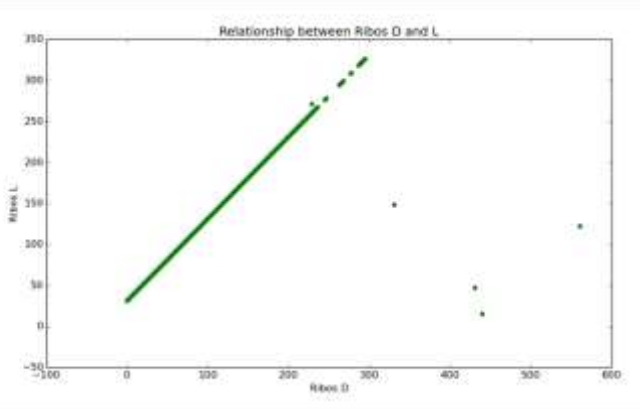

Fig. 6: DNA sequence relations between ribos D and L isolates

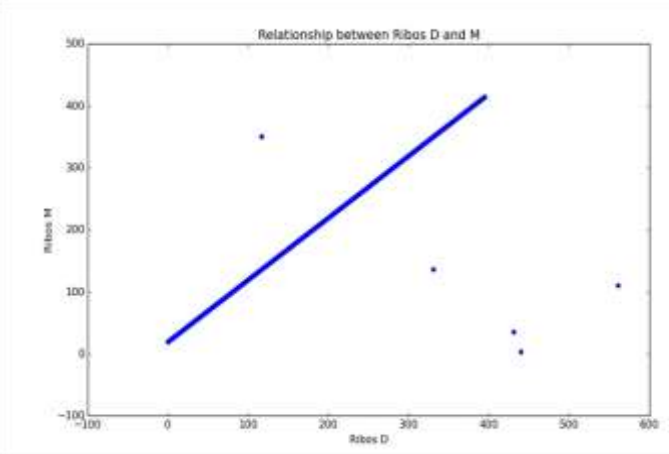

Fig. 7: DNA sequence relations between ribos D and M isolates

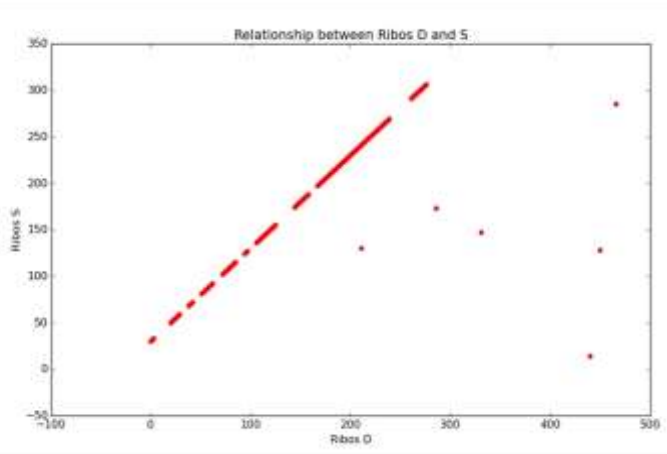

Fig. 8: DNA sequence relations between ribos D and S isolates

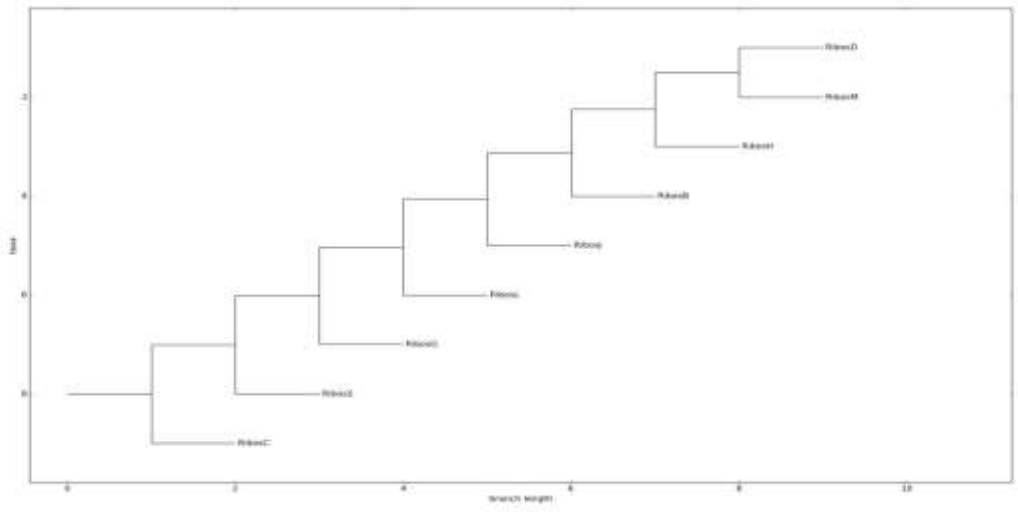

Fig. 9: Genetic relatedness of *Bacillus* species isolated from ready-to-eat (RTE) foods

Print ISSN: 2397-7728, Online ISSN: 2397-7736

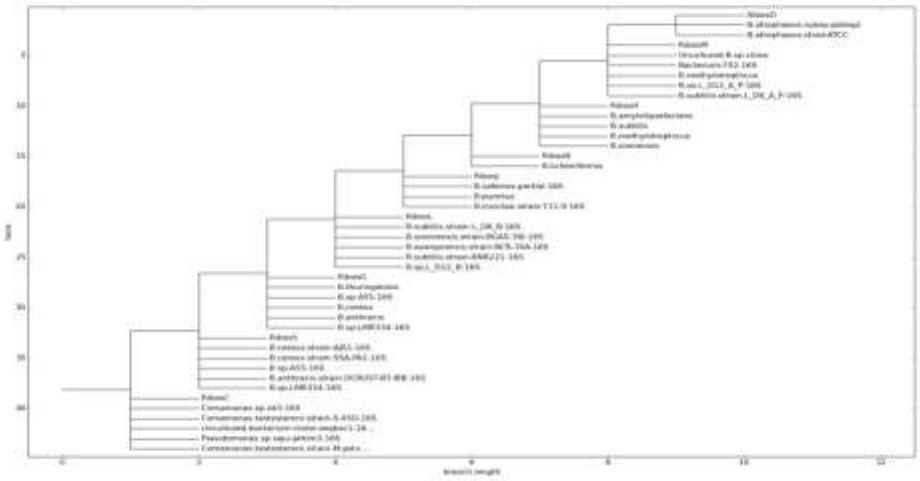

Fig. 10: Phyletic relatedness of isolated *Bacillus* species and database closely related sequences

DISCUSSION

In the classical identification approach; appropriate growth characteristics, colonial appearance and Gram stain of the culture, if demonstrated, covers for presumptive identification. However, it has been recommended that confirmation of identity requires more specific tests be done and commercial identification kit results and/or the reference laboratory reports be cross referenced (PHE, 2014). HiCrome *Bacillus* agar is a chromogenic medium used in this study, and is useful for detection of β-glucosidase activity of *Bacillus* microorganisms (Němečkova *et al*., 2011). Tallent *et al.* (2012) also looked at the characteristic appearance of these microbes on this and other chromogenic media. It is worthy of note that while the classical/conventional method identified all isolates as a member of a particular species in the *Bacillus* genus, in about 33% (3 of 9 identified cases, that is with ribos B, C and G) a discordance was observed. The ribos C microbe was identified as *B. subtilis* in the classical method, but as a member of the *Comamonas* sp. using the PCR method. Also, the ribos B microbe was identified as *B. cereus* under the classical scheme, but as *B. licheniformis* strain under the PCR. This once again reiterates the presumptive nature of identifying members of this genus using morphological and biochemical characteristics alone, and without recourse to more recent, advanced and sensitive molecular methods (Marston *et al.*, 2006; PHE, 2014). However, the PCR method provided results in agreement with the phenotypical, classical and genotypical identification more than 70% of the time. This method seemed to be more sensitive and precise in the identification of *Bacillus* species/strains. This aligns with the submission of Manzano *et al*. (2003).

The *Bacillus* genus comprises a heterogeneous group whose taxonomic rearrangement at the generic level have led to splitting of the genus, a process that is still on-going. This makes the genus an interesting group of bacteria for taxonomic and identification studies. According to the 'List of Prokaryotic Names with Standing in Nomenclature' 143 unique and valid *Bacillus* species are currently described (Slabbnick *et al*., 2008). As approved by the ad hoc committee

Print ISSN: 2397-7728, Online ISSN: 2397-7736

for the re-evaluation of the species definition in bacteriology, the current definition for a bacterial species is 'a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions'. The committee concluded that the standard for species delineation is a DNA-relatedness as measured by DNA-DNA hybridization of 70% or more (Stackebrandt *et al*., 2002). It should however be noted that species with a DNA-relatedness of more than 70% usually also have a 16S rRNA sequence similarity of more than 97%. Nonetheless, researchers have stated that bacterial taxa should be delineated polyphasically through a consensus based on both phenotypic and genotypic information. In this study, we have been able to establish some genomic relation between a genus of different species of microorganisms using the BioPython programming language. Having compared the sequences of the species, it was illustrated that a close relationship exists between the different species of the *Bacillus* genus (Fig. 1-8). Comparison of 16S rDNA sequence between the isolated bacteria showed that species were very homogeneous, with very few nucleotide differences. This finding suggests that colonization of sample sites were most probably from a common source. This may have occurred from the food handlers, air or as environmental contaminants from where these foods were prepared. This also points to the ubiquity of members of this genus and their ability to survive in a wide range of environments. Phylogenetic analysis based on 16S rDNA sequences indicated that most of the isolated bacteria belonged to the genus *Bacillus*. A rooted phylogenetic tree is given in Fig. 9. Ribos D (*Bacillus atrophaeus*) cluster with Ribos M (Uncultured *Bacillus* sp.) isolate, indicating a close genotypic relationship between the two isolates. Ribos S (*Bacillus cereus*) and Ribos C (*Comamonas* sp.) isolate branched off farthest, with least genetic relatedness. Both microorganisms were from different sources and belong to difference genus. All bacilli isolates branch of separately from a single taxa as different species of different strains within a group of the *Bacillus* genus, indicating singularity in identities which may be based on difference(s) on only one or more characteristics biochemically and/or genetically as opined by Khataminezhad *et al*. (2014), Priya and Vasuki (2014), and Amin *et al*. (2015). The NCBI database sequence that cluster most closely to the original isolate nucleotide sequences were taken as the best identity for the RTE food isolates as outlined in tables 1 and Fig. 10.

CONCLUSION

This study has demonstrated that some of the popular types of ready-to-eat foods that are sold on the streets are contaminated with *Bacillus cereus* and other *Bacillus* species. This work puts forward the need to make test for *Bacillus* species part of normal routine checks and analyses for ready-to-eat foods. It also reiterates the need for use of more sensitive and precise methods of identification for this group of microorganisms. Members within the *Bacillus* genus are closely related molecularly. While classical methods are fast becoming obsolete, the use of methods such as the analytical profile index kit and the PCR/sequencing method, among others, are highly recommended for the identification of *Bacillus* species. Hence, we join in reaffirming a stance as submitted by earlier researchers that classical schemes often do not allow the identification of atypical and intermediate strains. It is also widely agreed that there is considerable room for improvement in the taxonomy of the genus, *Bacillus*. It is also particularly important that a study of new isolates within this genus would require more sensitive and efficient methods of identification. Even now, as well as in the future, species/strain-specific method should be continually developed and tailored to identify particular strains within the *Bacillus* genus.

Print ISSN: 2397-7728, Online ISSN: 2397-7736

REFERENCES

Amin, M., Rakhisi, Z. and Ahmady, A. Z. (2015). Isolation and identification of *Bacillus* Species from soil and evaluation of their antibacterial properties. *Avicenna J. Clin Microb. Infec*., 2(1): e23233.

Cheesebrough, M. (2006). District laboratory practice in tropical countries. Cambridge University press, UK. Second low price edition. Pg 434.

Claus, D. and Berkeley. R. C. W. (1986). Genus *Bacillus*. In Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore. Pp. 1105-1139.

Drobniewski, F. A. (1993). *Bacillus cereus* and related species. Clinical Microbiology, 6: 324-338.

Ehling-Schulz, M., Svensson, B., Guinebretiere, M. H., Lindback, T., Andersson, M., Schulz, A., Fricker, M., Christiansson, A., Granum, P. E., Martlbauer, E., Nguyen-The, C., Salkinoja-Salonen, M. and Scherer, S. (2005). Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. *Journal of Microbiology*, 151: 183– 197.

European Food Safety Authority (EFSA). (2005). EFSA Opinion. Reducing risk on *Bacillus cereus* to food chain, 2005– Internete: http://www.foodnavigator.com/Legislation/Reducingrisk- on-*Bacillus-cereus*-to-food chain-EFSA-opinion.

Fawole, M. O. and Oso, B. A. (2001). Laboratory Manual of Microbiology. Spectrum books limited, Ibadan, Nigeria. Pp. 127.

Gilbert, R. J., Turnbull, P. C. B, Parry, J. M. and Kramer, J. M. (1981). *Bacillus cereus* and other *Bacillus* species: their part in food poisoning and other clinical infections. In The Aerobic Endospore-forming Bacteria: Classification and Identification. London: Academic Press; Pp 297–314.

Gordon, R. E., Haynes, W. C. and Pang, C. H.-N. (1973). The Genus *Bacillus.* Washington, DC: United States Department of Agriculture.

Granum, P. E. and Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *Federation of European Material Societies Microbiology* Letters, 157: 223–228.

Khataminezhad, M. R., Nuhi, A., Razavi, M. R., Nejadsattari, T. and Nazemi, A. (2014). Isolation and molecular identification of extracellular lipase-producing bacillus species from soil. *Annals of Biol. Res.*, 5 (1):132-139.

Kramer, J. M. and Gilbert, R. J. (1989). *Bacillus cereus* and other *Bacillus* species In M.P. Doyle (Ed). Food borne Bacterial Pathogens. New York: Marcel Dekker; Pp 21–70.

Manzano M., Cristina C., Mędrala D., Antoni C. and Comi G. (2003). Optimization of DNA extraction to detect *Bacillus cereus* from food using a PCR technique – short communication. *Polish J. of Food Nutr. Sci.*, 53: S69–S71.

Marston, C. K., Gee, J. E., Popovic, T. and Hoffmaster, A. R. (2006). Molecular approaches to identify and differentiate *Bacillus anthracis* from phenotypically similar *Bacillus* species isolates. *BMC Microbiology*, 6:22.

Mugg, P., Seymour, S. and Clark, S. (2013). A New Method for the Identification of *Bacillus* spp. and Related Species Involved in Food Poisoning and Spoilage. Microgen Bioproducts Ltd, Camberley, Surrey, UK.

Němečkova I., Solichova K., Roubal P., Uhrova B. and Švirakova E. (2011). Methods for detection of *Bacillus* sp., *B. cereus*, and *B. licheniformis* in raw milk. *Czech J. Food Sci.*, 29 (Special Issue): S55–S60.

Print ISSN: 2397-7728, Online ISSN: 2397-7736

Olutiola, P. O., Famurewa, I. and Sonntag. H. G. (1991). An Introduction to General Microbiology. A practical approach: HeidelbergerVerlagsansalt und DruckerelGmbH. Heidelberg.

Priest, F. G. (1981). DNA homology in the genus *Bacillus.* In: Berkeley R. C. W. and Goodfellow M. The Aerobic Endospore-forming Bacteria. Classification and Identification. London: Academic Press; Pp. 33-57.

Priya, R. and Vasuki, R. (2014). Screening and molecular characterization of Bacillus sp PR01 for the production of protease enzyme. *Int. Res. J. Pharm.*, 5(3): 191-194.

Public Health England. (2014). Identification of *Bacillus* species. UK Standards for Microbiology Investigations. ID 9 Issue 2.3.

Rusul, G. and Yaacob, N. H. (1995). Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA. *International Journal of Food Microbiology* 25: 131-139.

Shinagawa, K. (1993). Serology and characterization of *Bacillus cereus* in relation to toxin production. Bulletin of the *International Dairy Federation* 287: 42-49.

Slabbinck, B., De Baets, B., Dawyndt, P. and De Vos, P. (2008). Genus-wide *Bacillus* species identification through proper artificial neural network experiments on fatty acid profiles. *Antonie van Leeuwenhoek*, 94:187–198.

Sneath, P. H. A. and Collins, V. G. (1974). A study in test reproducibility between laboratories: report of a *Pseudomonas* working party. *Antonie van Leeuwenhoek*; 40:481-527.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Ka¨mpfer, P., Maiden, M. C. J., Nesme, X., Rossello´-Mora, R., Swings, J. and Tru¨per, H. G. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int. J. Syst. Evol. Micr*., 52:1043–1047.

Tallent, S. M., KoTewicz, K. M., Strain, E. A. and Bennet, T. R. W. (2012). Food Biological Contaminants. Efficient Isolation and Identification of *Bacillus cereus* Group. *Journal of AOAC International*. Vol. 95(2):446-508.

Todar, K. (2009). The Microbial World Lectures in Microbiology. *http://textbookofbacteriology.net/ themicrobialworld/B.cereus.html.* Department of Bacteriology. University of Wisconsin-Madison.

Print ISSN: 2397-7728, Online ISSN: 2397-7736