

Molecular Identification of *Bacillus* species in Commercial Honey

Abstract

Aims: Honey is consumed by many people because of it has high value in maintain good health and in the treatment of various diseases. The problems encountered by honey producers is deterioration in quality with storage due to fermentation and support the growth of microorganisms. Based on this the microbiological quality of honey in retail outlets and open markets should be monitored properly. This study characterized and identified *Bacillus* species in honey using molecular and biochemical methods.

Study Design: An experimental design

Place and Duration of Study: The study was carried out in the department of Molecular Biology of the Nigerian Institute of Medical Research for the period of 8 weeks.

Methodology: A total of 40 organisms were isolated from 33 honey samples using the spread plate method. The organisms were identified using morphological and biochemical methods. The organisms were further identified using polymerase chain reaction.

Results: The study revealed that 14 out of the 40 organisms were confirmed to be *Bacillus* species.

Conclusion: PCR technique is more accurate and reliable method of identification than morphological and biochemical test.

Key words: *Bacillus* species, Biochemical test, Honey, Polymerase Chain Reaction

26 1. INTRODUCTION

27 Honey is a natural sweet substance produced by *Apis mellifera* bees from the nectar of
 28 plants [1]. Honey has been used in most culture for the healing of wounds, burns,
 29 rashes and ulcers of every kind for thousands of years. This has led current scientists
 30 in the last few years to study the properties and the therapeutic effects of honey,
 31 these studies have confirmed the use of honey due to its natural components. Based
 32 on this information scientists focused their attention on the differences, benefits and
 33 effects found among honeys from various countries[2]. Honey contains *bacillus*
 34 which are aerobic spore-forming bacteria that are found in honey as spores and they
 35 survive in honey for long periods withstanding heat and other thermal application.
 36 The quality of honey is usually affected most times by microorganisms present in
 37 honey. The antibacterial activity of honey plays a role in reducing spore formers to
 38 the minimal population in honey. This is not guaranteed due to adulteration and
 39 mode of preparation of the product in some regions.

40 The use of honey as a wound dressing agent has been argued against because of the
 41 risk of it possibly causing botulism. Microorganisms that survive in honey are those
 42 that withstand the concentrated sugar, acidity and antimicrobial characters of honey.
 43 *Bacillus* species are most prevalent in honey, followed by Gram- variable pleomorphic
 44 bacteria, mould, actinomycetes. Gram negative rods (Enterobacteriaceae) have been
 45 isolated from bee larva, thereby having the possibility of being found in honey.
 46 There is a risk of introducing spores into wounds if honey is used as a dressing. For
 47 honey to be used as dressing it has to be sterilized to get rid of all the spores
 48 [3]. Honey researchers in Nigeria have studied the antimicrobial, antioxidant and
 49 physicochemical parameters of various honeys[3, 4, 5, 6], scanty research has been
 50 done on molecular studies of the microorganisms found in honey. The traditional
 51 basis for the identification of bacterial organisms in food or the environment has to
 52 do with their isolation or propagation in the laboratory, where biochemical,
 53 morphological and serological tests are used in identification. The genus *Bacillus* sp.

is ubiquitous, endospore- forming Gram- positive bacteria. Their primary habitat is soil and due to resistant spores they can colonize different environments such as honey, food, animals, insects e.t.c ([7, 8]. *Bacillus* species has a wide distrubtion in nature, they are frequently isolated from plants, but they are also well adapted for growth in the intestinal tract of insects such as bees and animals[8]. *Bacillus* has been isolated from food borne illness out breaks, it is also present in stools of healthy humans.

Molecular techniques have enabled researchers to identify microorganisms more concisly. Molecular studies have shown that greater number of bacterial species in samples and specimens than culture results shows. The continous use of culture and molecular techniques for the identification of microorganisms is necessary to compare and contrast these newer methods to traditional methods of identification[8].

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 33 honey samples were used for this study. Twenty- eight of the honey samples were of local origin while 5 were of international origin. Twenty-eight commercial honey samples were obtained from the six geographical regions in Nigeria. Four honey samples were obtained from North- Central (Jos, Kogi), two from North – East (Jalingo), and two from North – West (Kaduna). Ten honey samples were obtained from South – East (National Root Crop Research Institute Umudike (NRCRI), Nsukka, Owerri). Eight honey samples were obtained from South- South (Warri, Yenegoa, Port Harcourt and Calabar) and two honey samples from South- West (Oyo) which was obtained from International Institute of Tropical Agriculture, Ibadan (IITA).

2.2 Isolation and Identification of Bacteria from honey samples

A drop of honey was inoculated using a sterile spreader into Nutrient Agar, Blood Agar and Tryptone Soy Agar plates, plates were incubated for 24hrs at 37°C. The colony on the plates was sub cultured onto Nutrient Agar to obtain pure colony. Pure colonies were stored and identified using standard microbiological and biochemical methods [9, 10].

2.3 Primer selection

The primer was selected through the combined use of four universal primers, XB4 employed as a 21 – oligonucleotide reverse primer, 5' –GTG TGT ACA AGG CCC GGG AAC-3' and 5' -CAG ACT CCT ACG GGA GGC AGC AGT- 3', the primers amplify a 598 – base pair PCR amplicon. XB4 was located at base positions 1324-1304, in relation to *Stenotrophomonas maltophilia* (GenBank Accession number AY169434). The primer used for this study was selected based on a previous study with slight modifications [11].

2.4 PCR protocol using isolates from honey

All isolates were sub cultured into Muller Hilton Agar. Genomic DNA was extracted from a single colony with ZR Fungal/ Bacterial DNA MiniPrep™ (ZYMO RESEARCH) purity kit. All the reaction mixes were set up in a PCR hood in a separate room from which the DNA was extracted and also the amplification and post- PCR room to minimise contamination in accordance with Millar *et al* 2002 laboratory guidelines. PCR was performed in 25µl of a reaction mixture containing DNA (10-200ng), 200µM of deoxynucleoside (d NTP) (Promega), 1.5 mM MgCl₂, 1X PCR buffer, 20 pMol (each) of primers, 1 unit of Taq polymerase (Promega) and sterile distilled water. Following a hot start, the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermo cycler: 96°C for 3min followed by 40 cycles of 96°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by 72°C for ten minutes. The amplification product was separated on 1.5%

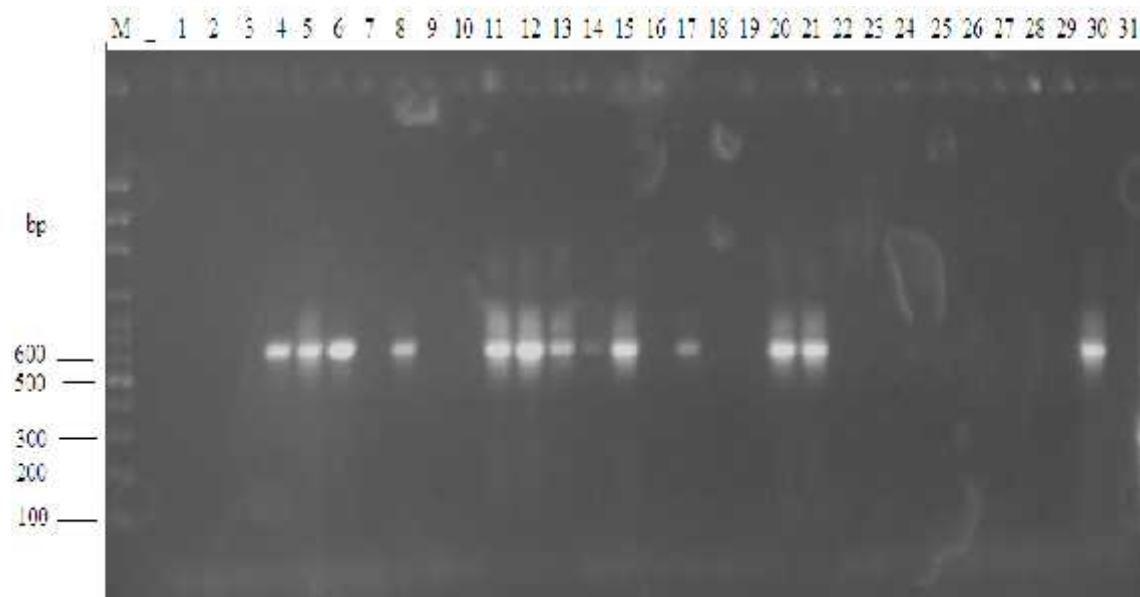
106 agarose gel electrophoresis and visualized by ethidium bromide staining. One
107 hundred (100) bp DNA ladder was used as DNA molecular weight standard [12].

108 3. RESULTS AND DISCUSSION

109 Morphological studies of the isolates from the honey samples revealed that most of
110 the isolates were Gram positive spore formers and just few were not spore formers.

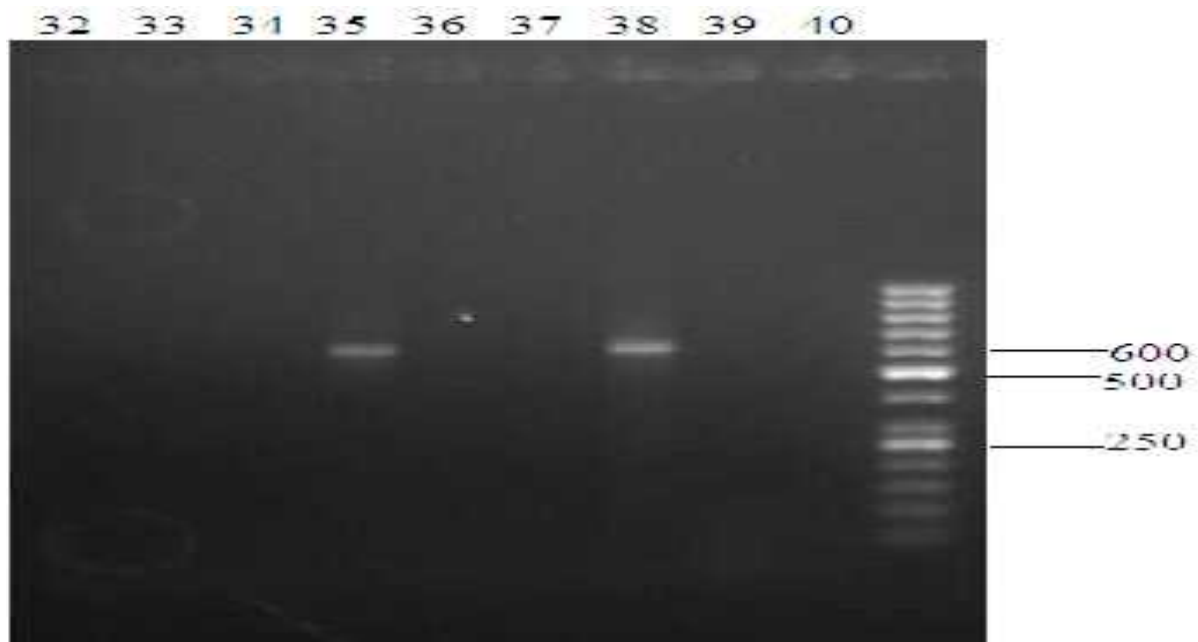
111 Biochemical test revealed that isolates were catalase positive and produced starch
112 hydrolysis. Oxidase test, hydrogen sulphide fermentation was also
113 negative. Morphological and biochemical identification showed that out of the forty
114 isolates three were identified as *Micrococcus* sp. And two were identified as
115 *Eubacterium* sp. The microbial load of the honey samples were observed to be high.

116 The results of PCR are represented in Figures 1 and 2, a total of 40 isolates obtained
117 from the selected honey samples were subjected to PCR. About 80% of these
118 isolates had colonial appearance resembling *Bacillus* spp. PCR amplifications on high
119 quality Genomic DNA preparations of these isolates generated amplicons of the
120 same size for some of the isolates. Out of the forty isolates 14 generated amplicons
121 4,5,6,8,11,12,13,15,17,20,21, 30-35 and 38, these amplicons were quite similar. This
122 shows the remaining 27 isolates were not *Bacillus* species. These isolates are:
123 1,2,3,7,9,10,14,16,16,18,19,22,23,24,25,26,27,28,29,31,32,33,34,36,37,39 and 40; as
124 represented in figures 1 and 2. The overall results revealed that 35% of organisms in
125 honey are spore formers representing *bacillus* species.



M=100bp

Fig. 1: Gel electrophoresis of the PCR amplified products for the detection of *Bacillus* species Well A 1 to 31



100bp

Fig. 2: Gel electrophoresis of the PCR amplified products for the detection of *Bacillus* species for well B 32 to 40

The rapid advancement in molecular biology over the last 20 years has provided new techniques that are aimed at assisting in the detection and identification of micro-organisms from various sources. With the advent of molecular biology, microbiologists have another avenue to detection and identification of microorganisms. Polymerase chain reaction is a technique which uses a DNA polymerase enzyme to make a huge number of copies virtually any given piece of DNA or gene.

The detection of the microbiological quality of honey in retail outlets and open markets should be properly monitored regularly. This should be done because the presence of high microbial population in honey indicates the non-hygienic condition and lack of safety of the product been used to treat various ailments and for consumption especially when children under 12 months are involved [13, 14].

The identification of bacillus species such as *Paenibacillus* in honey is very important because some of these species of *bacillus* can cause disease such as American foulbrood. American foulbrood is a disease of high socioeconomic importance and significance in international trade. The spores are highly resistant to heat and chemical agents and can survive in the environment for several years. Only the spores are capable of inducing the disease. Using a biological method to isolate these organisms requires a complex media for growth which are readily available in some laboratories. DNA –based identification methods such as 16S rRNA gene sequencing and 16S- 23S intergenic region sequencing have been used widely for the purpose of identification and typing of microorganisms isolated from honey, natural environments including fermented foods in some part of the world[15].

Unfortunately, no molecular identification has been carried out on the organisms isolated from Nigerian honey, this work was a preliminary study on the identification of bacteria associated with Nigerian honey using the morphology and biochemical methods which are frequently used in Nigeria and compared to PCR screening.

Bacillus species has a wide range of distribution in nature, they are frequently isolated from plants, but they also have a well adapted for growth in the intestinal tract of insects and animals[7]. It has been isolated from food that were involved in food borne illness outbreaks, *bacillus* species are also present in stools of healthy humans.

A study carried out in Argentina involving 433 honey samples, the work showed 27% *B. cereus*, 14% yielded other *bacillus* species. The *B. cereus* obtained from Argentinean honey were compared with isolates from honey from other countries using rep-PCR fingerprinting with primers Box, REP and ERIC, restriction fragment length polymorphism analysis of a 16S RNA gene fragment and morphological and biochemical tests. The result showed high diversity, both phenotypic and genotypic among the isolated *B. cereus*[16]. The results obtained in this study is also in accordance with studies carried out by Lurlina and his colleagues ~~which~~ revealed the presence of *Bacillus* species in 28 honey samples out of 70 [17].

For organisms to be identified correctly different methods have to be used to confirm the results. Identification based on rRNA gene sequence fails to distinguish one species from the other if they share highly similar rRNA genes, other genes such as *recA* and DNA has been employed instead of rRNA genes[18, 14]. It is therefore necessary to compare other methods of identification before concluding.

4. CONCLUSIONS

The study revealed that honey has the ability to support the growth of microorganisms even as it has antimicrobial activity. These microorganisms associated with honey cannot be identified by ~~biochemical methods alone~~. Current methods for molecular identification of microorganism should be carried out alongside ~~biochemical~~ methods of identification.

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