

Preliminary Detection of *Bacillus* species in Commercial Honey

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Abstract

Aim: This research is aimed at detecting the presence of *Bacillus* species in honey using biochemical tests and preliminary molecular studies.

Methodology: 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 from commercial retailers. The five foreign honey samples were obtained from the supermarkets namely: friz fruit, Blossom, Forever, Aloe Vera and Rose honey all of international origin. The honey samples were inoculated into sterile agar plates using the spread plate technique. Isolates obtained were purified and subjected to biochemical tests and further identification using polymerase chain reaction.

Results: All the honey samples had microbial growth in them, higher counts were observed in the commercial honeys from retailers than the foreign honey samples. Forty isolates suspected to be *Bacillus* from biochemical tests were subjected to PCR, 14 from the 40 were confirmed to be *Bacillus* species.

Conclusion: Microorganisms in honey cannot be identified correctly with morphological and biochemical examinations alone, but combine use of morphological, biochemical tests and PCR technique is more accurate and reliable method of identification.

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

1. INTRODUCTION

Honey is a natural sweet substance produced by *Apis mellifera* bees from the nectar of plants [1]. Honey has been used in most culture for the healing of wounds, burns, rashes and ulcers for years now. This has led current scientists in the last few years to study the properties and the therapeutic effects of honey, these studies have confirmed the use of honey due to its natural components. Based on this information scientists focused their attention on the differences, benefits and effects found among honeys from various countries [2]. Honey possesses antibacterial activity against gram positive and negative organisms

thus reducing the number of organisms that can grow in honey. Organisms found in honey come from the nectar, pollen, processing area, machines and containers that are not properly washed [19, 20, 24]. Honey contains *Bacillus* which are aerobic spore-forming bacteria that are found in honey as spores and they survive in honey for long periods withstanding heat and other thermal application. The quality of honey is affected most times by microorganisms present in honey. The antibacterial activity of honey plays a role in reducing spore formers to the minimal population in honey. This is not guaranteed due to adulteration and mode of preparation of honey in some regions [13, 22].

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The use of honey as a wound dressing agent has been argued against because of the risk of it possibly causing botulism. Microorganisms that survive in honey are those that withstand the concentrated sugar, acidity and antimicrobial characters of honey. *Bacillus* species are most prevalent in honey, followed by Gram- variable pleomorphic bacteria, mould, actinomycetes. Gram negative rods (Enterobacteriaceae) have been isolated from bee larva, there by having the possibility of been found in honey. Studies have shown the following Gram negative bacteria found in honey are *Achromobacter*, *Citrobacter*, *Enterobacter*, *Ewinia*, *Escherichia coli*, *Flavobacterium*, *Proteus* and *Pseudomonas*. There is a risk of introducing spores into wounds if honey is used as a dressing without been certain of the quality and source of the honey. Honey researchers in Nigeria have studied the antimicrobial, antioxidant and other parameters of various honeys [3, 4, 5, 6], scanty research has been done on molecular studies of the microorganisms found in honey from Nigeria. 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 distribution 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].

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 continuous use of culture and molecular techniques for the identification of microorganisms is necessary to compare and contrast these newer to modern techniques of identification [8].

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2. MATERIALS AND METHODS

3.

3.1 Sample Collection

A total of 33 honey samples was used for the study, out of the 33 twenty-eight were commercial honey produced locally in various states in Nigeria. They were obtained from

commercial honey retailers. Five processed commercial honey samples of foreign origin were purchased from the supermarkets (blossom, Aloe Vera, Forever, Rose and frizfruit honey). 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 were obtained from International Institute of Tropical Agriculture, Ibadan (IITA).

2.2 Isolation and Identification of Bacteria from Honey Samples

One hundred micro liters of honey samples was spread on nutrient agar plates (Oxoid), Blood Agar and Tryptone Soy Agar (Oxoid) plates. Plates were incubated for 24 hrs at 37°C. The colonies on the plates were sub cultured onto nutrient agar plates to obtain pure colony. Pure colonies were stored and identified using standard microbiological and biochemical methods [9, 10].

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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 AY 169434). 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 Hinton Agar (Oxoid). 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 minimize contamination in accordance with Millar *et al* 2002 laboratory guidelines. PCR was performed in 25µl of a reaction mixture containing DNA (10-200ng), 2µl of deoxynucleoside

(d NTP) (Promega), 1.5 mM MgCl₂, 1X PCR buffer, 1 µl (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% agarose gel electrophoresis and visualized by ethidium bromide staining. One hundred (100) bp DNA ladder (Promega) was used as DNA molecular weight standard [12].

3. RESULTS AND DISCUSSION

Morphological and biochemical examination of the isolates from the honey samples revealed that 70% of the isolates were Gram positive spore formers and 30% were non spore formers as shown in Table 1 below. Biochemical test revealed that the isolates were catalase positive and produced starch hydrolysis. Oxidase test and hydrogen sulphide fermentation were negative. Morphological and biochemical identification showed that out of the forty isolates tested 3 were identified as *Micrococcus* sp. 2 were identified as *Eubacterium* sp. and rest 35 were suspected to be *Bacillus* species. The remaining 35 were further subjected to PCR technique, fourteen out of the 35 were confirmed to be *Bacillus* species. Results obtained for the PCR are represented in Figures 1 and 2 below. Eighty percent of these isolates had colonial appearance resembling *Bacillus* spp. PCR amplifications on high quality Genomic DNA preparations of these isolates generated amplicons of the same size for some of the isolates. The 14 isolates that generated amplifications 4, 5, 6, 8, 11, 12, 13, 15, 17, 20, 21, 30, 35 and 38, these were quite similar. This shows that the remaining 21 isolates were not *Bacillus* species. They could be other organisms that can survive in honey and have morphological and biochemical characteristics resembling *Bacillus* species. These isolates were 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 represented in figures 1 and 2. The overall results revealed that 35% of organisms in honey are spore formers representing *Bacillus* species.

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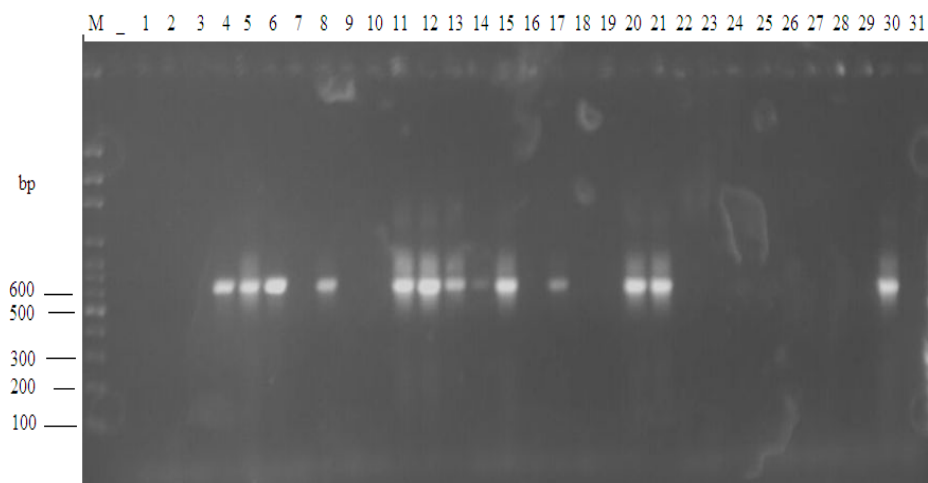
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Table 1. Characterization and Identification of Bacteria Isolated from Honey Samples

S/NO	Isolate codes	Gram reaction	Catalase test	Methyl Red	Voges Proskauer	Indole production	Citrate test	Oxidase test	Motility test	Starch Hydrolysis	Spore stain	Glucose	Lactose	Mannitol	Xylose	Galactose	Identified isolate
1	R1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
2	R2	+rod	-	+	+	-	+	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
3	R3	+rod	-	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
4	C1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
5	C2	+rod	+	+	+	-	-	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
6	C3	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
7	C4	+cocci	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Micrococcus</i> sp
8	N1	+rod	-	-	+	-	-	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
9	N2	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
10	N3	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
11	E1	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
12	E2	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
13	E3	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp
14	I1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Eubacterium</i> sp.
15	I2	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
16	FE1	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
17	FE2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
18	U1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
19	U2	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
20	U3	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
21	U4	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
22	F3	+cocci	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Micrococcus</i> sp.
23	Ma	+cocci	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Micrococcus</i> sp.
24	Mb	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
25	D1	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
26	D2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
27	K1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.

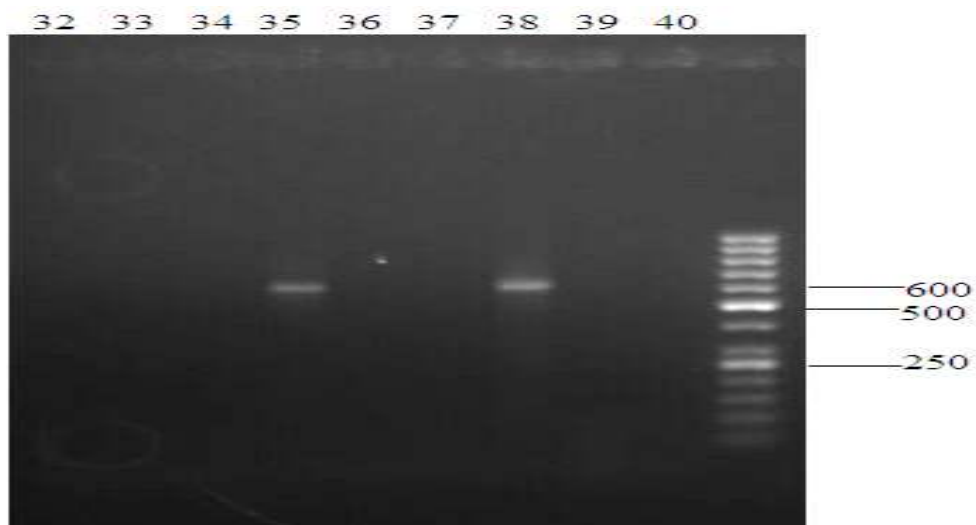
28	K2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
29	K3	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
30	IB1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
31	IB2	+cocci	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Eubacterium</i> sp.
32	B3	-rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
33	E1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
34	M1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
35	M2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
36	M3	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
37	BL1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
38	BL2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
39	BL3	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.
40	BL4	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	<i>Bacillus</i> sp.

+ = Positive - = Negative



M=100bp

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



100bp

Fig. 2: Gel electrophoresis of the PCR amplified products for the detection of *Bacillus* species.

In most institutions in developing countries, culture technique and biochemical test are methods used for the identification of microorganisms from different sources. These methods are used because it is cheaper to afford and easy to come by. Culture technique and biochemical tests are time consuming, results obtained most times are not always accurate because isolates are prone to contamination and the reagents used most times are expired.

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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 for 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.

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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 biolog method to isolate these organisms requires a complex media for growth which are expensive and 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 [15]. Unfortunately, no molecular identification has been carried out on the organisms isolated from Nigerian honey, this work was a preliminary study on the detection of *Bacillus* spp of in Nigerian honey using morphological and biochemical methods which are frequently used in Nigeria and compared to PCR screening.

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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]. Esawy and his colleagues identified 99% of *Bacillus* spp. in honey by morphological examination and molecular biology analysis [20, 23]. This is in conformity with the study carried out by Sadik et al that confirmed the presence of microorganisms ranging

from bacteria, moulds and yeasts in honey. Their study was carried out using morphological appearance of such isolate on specialize media [21].

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 who revealed the presence of *Bacillus* species in 28 honey samples out of 70 [17]. For organisms in honey to be identified correctly different methods involving morphological examination, biochemical and molecular studies have to be used to get accurate 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 [14, 18]. It is therefore necessary to compare other methods of identification before concluding.

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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 morphological examination and biochemical methods of identification.

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