Journal of Applied Life Sciences International
5(4): 1-6, 2016; Article no.JALSI.24759
ISSN: 2394-1103

SCIENCEDOMAIN *international*
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Molecular Characterization and Identification of *Bacillus* spp. MLSU-PSSKB Isolated from Fecal Contents of *Pteropus giganteus* in Udaipur, Rajasthan, India

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Authors' contributions

This work was carried out in collaboration between all authors. Authors PS and SKB designed the study, wrote the protocol and authors PS and SKB wrote the first draft of the manuscript. Author SKB monitored the experimental procedures and author PS handled the critical revision of the manuscript. Authors DKS and AP managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JALSI/2016/24759
Editor(s):
(1) Yunjun Yan, Institute of Bioenergy and Ecology, College of Life Science and Technology, Huazhong University of Science and Technology, P. R. China.
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Reviewers:
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(4) Guadalupe García-Elorriaga, Mexican Social Security Institute, Mexico.
Complete Peer review History: http://sciencedomain.org/review-history/14924

Original Research Article

Received 31st January 2016
Accepted 25th May 2016
Published 7th June 2016

ABSTRACT

Introduction: The *Bacillus* genus, being the largest, consists of Gram-positive, endospore-forming, chemoheterotrophic rods which are usually motile and peritrichously flagellated. Many species, of *Bacillus* are of considerable economic importance as these serve as insecticides, cause food poisoning, produce antibiotics etc.
Aim: The aim of the present investigation was to assess and evaluate the possibility of *Pteropus giganteus* as a carrier of disease causing microorganisms. Hence in the present study an effort has

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been made to identify and characterize *Bacillus* species based on 16S rRNA from the faeces of *Pteropus giganteus* from Udaipur, Rajasthan India.
Its phylogenetic tree has also been derived, which shows evolutionary relationship of eleven related taxa. This is the first report from Indian subcontinent correlating the role of this megachiropteran as a carrier of *Bacillus* spp. MLSU-PSSKB.
Study Design: Roosting sites of *P. giganteus* was study site and sterile fecal sample was collected and subsequently cultured of *Bacillus* spp. was amplified and sequenced. The phylogenetic analysis of isolated genes from samples was also carried out in order to find their taxonomical status.
Results: The culture showed the presence of *Bacillus* spp. based on nucleotide homology and phylogenetic analysis its nearest homolog species was determined to be. 16S rRNA sequence of *Bacillus* spp. 124(FJ372768.1).The phylogenetic analysis indicated that the present sequence occurred in the same clade of FJ372768 with high bootstrap value. This novel *Bacillus* spp. was named "MLSU-BSPSSKB".
Conclusion: This present undertake investigation has categorically demonstrated that a novel species of *Bacillus* is prevalent in *P. giganteus* in Udaipur city, India, which may indicate their zoonotic importance.

Keywords: Pteropus giganteus; faeces; Bacillus spp. MLSU-PSSKB; phylogenetic tree.

1. INTRODUCTION

India is one of the world's richest countries in terms of its vast array of chiropteran diversity. Chiropterans are unique because of their evolutionary status, aerial habit, the diverse ecological habit and habitat and geographical regions in which they occur. Chiropterans belong to order Chiroptera of Class Mammalia. Chiropterans are classified in two suborders– Megachiroptera, which includes all the frugivorous bats and Microchiroptera that includes the insectivorous bats. Suborder Megachiroptera includes all frugivorous bats which are generally large in size. The suborder Megachiroptera consists of one family: Pteropodidae, inclusive of 170 species. Megachiropterans are characterized by large eyes, small ears, and dog-like snouts. Being frugivorous in nature they feed primarily on plant material that could be fruit, nectar or pollen. These bats also act as seed dispersers and pollinators. Udaipur city, of state Rajasthan, India, is a beautiful tourist city of international repute and is famous for its lakes and gardens. It is in these gardens, *Pteropus giganteus* resides and in the evenings, around and over these lakes a mass swarming takes place. They fly over the lakes; thousands in number and their fecal dropping are released in water resulting in its contamination. Therefore in the present research, it was thought to assess the role of *Pteropus giganteus* as a zoonotic agent for the spread of bacteria viz. [1-3] *Bacillus*. Genus *Bacillus* consisting of aerobic, Gram- positive or Gram variable, endospore-forming, chemoheterotrophic rods which are usually motile, was first described and classified by [4]. Many species of *Bacillus* are of considerable economic importance and have significant microbiological uses [5]. Numerous associated enzymes, antibiotics and other metabolites having medical, agricultural, pharmaceutical and other industrial applications have been derived from different *Bacillus* e.g. bacitracin by *B. licheniformis* or *B. subtilis*, polymyxin by *B. polymyxa* and gramicidin by *B. brevis*. *B. thuringiensis* is a known insecticide, *B. cereus* causes food poisoning and can infect humans, *B. anthracis* has been reported to be the causative agent of the disease anthrax, which affects mainly farm animals, are examples of few microorganisms that have adverse impact on humans and other organisms. Chiropterans mediated zoonoses has to be investigated, for these mammals can serve as major reservoirs for transmission of zoonotic agents other animals and humans [6,1,7,8]. In prior investigations, the role of chiropterans has been correlated with the transmission of a number of infectious agents such as rabies, Ebola virus, Hendra virus etc. [4,8-10]. However there is a paucity of similar information from Indian subcontinent, hence the present report has been an effort to elucidate the role of *P. giganteus* as a vector of zoonotic diseases. This study would help to generate a data base for assessing the transmission of certain microbial species. An effort has also been made to derive its phylogenetic tree.

2. MATERIALS AND METHODS

2.1 Sample Collection

The samples of feces were collected from Samor, Udaipur city of Southern Rajasthan India (lat_lon="24.0095 N 73.0137 E") at 5:30 PM in the month of March 2013.

The fecal Sample was collected in a special sterile box which was placed just beneath the tree. Care was taken to avoid contamination; hence the collected matter was placed immediately inside sterile plastic containers and was later processed in laboratory for subsequent culture. Some portion of faeces was also preserved in laboratory at 40C for further utilization.

2.2 Isolation of Bacterial Species

Fecal pellets were dissolved in soluble sterile TE buffers and cultured in nutrient medium in the laboratory using the kits which were purchased from Sisco Research Laboratory Pvt. Ltd. Mumbai, India (NM011 Nutrient Medium).

Serial dilutions of saline samples were prepared for bacterial isolation on nutrient agar media. For isolation, these were respectably incubated aerobically for 24 hours at 370C. After assessment of the samples from all media, on the basis of Gram staining, a total of 18 isolates were obtained by random selection. Identification of Bacterial species was done as per the techniques listed in [11,12]. The isolated microorganism on the basis of its morphology appeared to Bacilli which was further identified on the basis of molecular characterization.

2.3 Identification of Bacterial Species

The bacterial isolate was identified with 16S rRNA sequence and Bioinformatics analysis. Genomic DNA was isolated from the culture as per the technique of [13] its quality was evaluated on 1.2% Agarose Gel, where a single band of high-molecular weight DNA was observed. Fragment of 16S rRNA gene was amplified by PCR from the above isolated DNA. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers (5'TGCCCATCAGAGGGGGATAA3') and as reverse primer (5'GTGTCTCAGTCCC AGTGTGG3'). using BDT v3.1, cycle sequencing kit on ABI 3730xl genetic analyzer. Consensus sequence of 1271 bp 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the NCBI gene bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program and "Clustal W. Distance" matrix was generated using RDP database and the phylogenetic tree was constructed using the software, MEGA 4 [14].

2.4 Genomic DNA Extraction

Genomic DNA from all the isolates was extracted by the following method see paragraph Singh Preeti [15,9,10].

2.5 Molecular Phylogenetic Analysis of the Derived Species

2.5.1 Sequence analysis

The closest relatives of 16S rRNA sequences were determined by a search of the GeneBank DNA database using the BLAST algorithm [16]. Homology comparisons were performed using the Basic Local Alignment Search Tool (BLAST), online at the National Centre for Biotechnology Information (NCBI) homepage (http://www.ncbi.nlm.nih.gov). Identities of isolates were determined based on the highest score.

The 16S rRNA gene was sequenced and submitted to GeneBank with accession number KF777816. Further, the obtained sequence was compared with other related sequences to find most close homolog at NCBI using BLAST. The related 11 sequences were used to construct phylogenetic tree using the neighbor-joining method in order to find its exact taxonomic status [17]. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed [18]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method [19] and are in the units of the number of base substitutions per site Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were

eliminated from the dataset (Complete deletion option).There were a total of 1271 bp positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [14].

3. RESULTS AND DISCUSSION

The isolated culture was determined to be *Bacillus* spp. (Fig. 3) A single discrete PCR amplicon band of 1271 bp was observed when resolved on Agarose Gel (Gel Image Fig. 1). The obtained sequence showed highest percent identity (99%) with *Bacillus sp.* 124 (FJ372768.1), by NCBI, which was isolated from *Aedes aegypti* in Brazil. The percent identity of the present sequence with other closely related sequence has been presented in Table 1. The present sequence showed 92% and 93% identity with other *Bacillus* species reported worldwide. Further, the Phylogenetic tree was constructed to find its taxonomic status. In the tree, the isolated sequence of presented study fall in the same clade of *Bacillus sp.* 124 with 100 bootstrap value (Fig. 2) indicating that the isolated sequence was most close to *Bacillus sp.* 124. From the assessment of its phylogenetic tree it can be postulated that this bacterium has a diverse distribution for it has been isolated from hot springs, sludge's, paddy fields, vermicompost and mites [6,8]. However Some samples collected from Udaipur city were also negative for Bacillus.

1. DNA Marker 2. *Bacillus* spp.16S rDNA

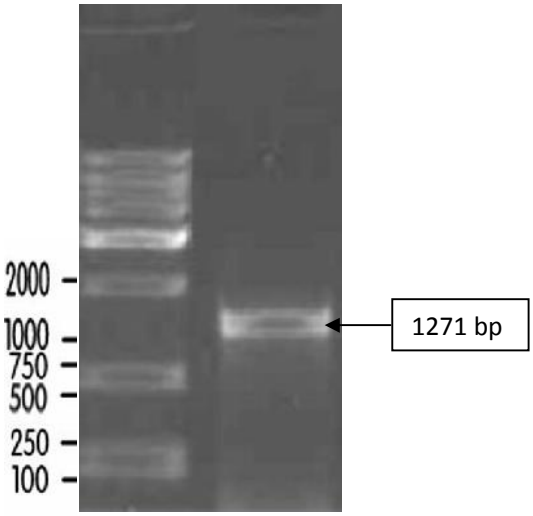

Fig. 1. Gel image of 16S rDNA amplicon

The arrow shows visualization of amplification 16S rDNA fragment isolated Bacillus spp. presence of 1271 bp
Lane 2:16S rDNA amplicon band
Lane 1: DNA marker

Table 1. Alignment view showing distance matrix based on nucleotide sequence homology of *Bacillus* spp. MLSU-BSPSSKB

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ372768.1	*Bacillus* sp. *I24*	2342	2342	100%	0.0	99%
GU136579.1	*Bacillus* sp. *S110(18)-1*	1838	1838	100%	0.0	93%
JN679100.1	*Uncultured Bacillus* sp. *clone 3.29m3*	1832	1832	100%	0.0	93%
JF346899.1	*Bacillus* sp. *QLPB08*	1816	1816	100%	0.0	92%
JX913830.1	*Bacillus koreensis strain Pb-WC11036*	1783	1783	100%	0.0	92%
JX566646.1	*Bacillus* sp. *6056*	1783	1783	100%	0.0	92%
JN993703.1	*B. koreensis strain TSI-2*	1783	1783	100%	0.0	92%
JN592473.1	*Bacillus* sp. *CRRI-56*	1783	1783	100%	0.0	92%
FJ006895.1	*Bacillus* sp. *WPCB111*	1783	1783	100%	0.0	92%
FJ006881.1	*Bacillus* sp. *WPCB065*	1783	1783	100%	0.0	92%

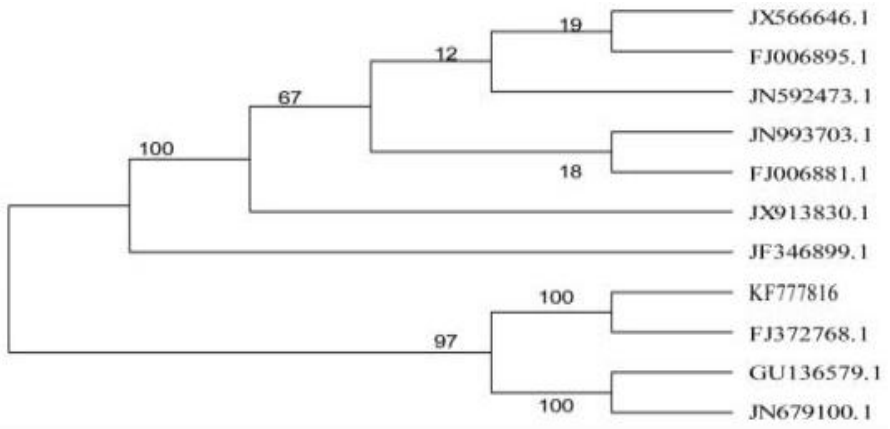

Fig. 2. Phylogenetic tree showing evolutionary relationship of 11 taxa

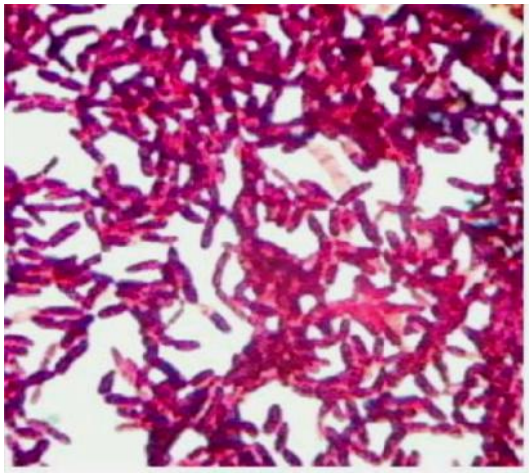

Fig. 3. Slide smear of *Bacillus* spp. *(100 X magnification)*

The smear shows the presence of occurring *Bacillus* spp. in the flagellate form stained with Gram's stain.

4. CONCLUSION

This present undertaken investigation has identified and characterized *Bacillus* spp. MLSU-BSPSSKB from the faeces of *P. giganteus* present in Udaipur city, Rajasthan, India and describes the presence and genetic characteristics of this species. This study also indicates that not all the organisms were carriers of this microorganism.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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International Journal of Current Microbiology and Applied Sciences. 2015; 4(3):143-149.
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Peer-review history:
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