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COMPARATIVE ANALYSIS OF 16SrRNA GENES OF *Klebsiella* ISOLATED FROM GROUNDNUT AND SOME AMERICAN TYPE CULTURE COLLECTIONS

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Abstract. Endophytic and associative bacteria like *Klebsiella* are known to enhance growth and yield of plants by fixing atmospheric nitrogen, solubilization of phosphate, production of phytohormones and siderophores. Despite this, less genetic information known concerning this important bacteria. Thus, this study was conducted to explore the relationship between *Klebsiella spp.* isolated from groundnut from Gadarif (Sudan) and some American type culture collections through *16SrRNA* gene analysis. Gadarif strain was isolated using Yeast Extract Mannitol Media, DNA was extracted, *16SrRNA* gene was amplified and sequenced. The results showed that *Klebsiella pneumoniae* from the American type culture collections was the most related to that isolated from Gadarif and *Klebsiella oxytoca* was the most different. The majority differences were found at several distinct positions. The study concluded that *Klebsiella spp*. isolated from different regions and different environmental conditions differ in their DNA sequences.
Keyword*:* Endophytic, groundnut, isolate, nucleotides, sequences.

Introduction

The genus, *Klebsiella*, named after the microbiologist Edwin Klebs, are characterized as rod–shaped, Gram–negative γ–proteobacteria that can live in water, soil, and plants and are pathogenic to humans and animals [PODSCHUN *et al*., 2001, BUTNARIU and BUTU, 2013].

Klebsiella planticola and *Klebsiella terrigena* were identified in 1981, and *Klebsiella ornithinolytica* was identified in 1989.

The first two species are associated primarily with botanical and aquatic environments, whereas the last species, formerly known as ornithine–positive *Klebsiella oxytoca*, was first identified from clinical samples [BAGLEY *et al*., 1981; IZARD *et al*., 1981; SAKAZAKI *et al*., 1989].

Klebsiella trevisanii [FERRAGUT *et al*., 1983] was combined to *Klebsiella planticola* on the basis of their extensive DNA±DNA homology [GAVINI *et al*., 1986].

Klebsiella pneumoniae and *K. planticola* strains have been isolated from diverse plants [BAGLEY *et al*., 1981; HAAHTELA and KORHONEN, 1985] including rice [LADHA *et al*., 1983] and maize [PALUS *et al*., 1996].

Pathogenic *Klebsiella* have been found to successfully colonize potato and lettuce [KNITTEL *et al*., 1977] and plants may be considered to be reservoirs of human opportunistic *Klebsiella*.

In plants, *K. pneumoniae* strains capable of living as endophytes are of interest as they can increase plant growth under agricultural conditions [RIGGS *et al*., 2001], and provide fixed nitrogen to certain grasses [SEVILLA *et al*., 2001; INIGUEZ *et al*., 2004].

Klebsiella strains may also be human pathogens contaminating the food supply.

In humans, certain strains of *K. pneumoniae* are known to cause nosocomial urinary tract infections, and pneumonia, leading to septicemia and death [FOUTS *et al*., 2008].

The approach of comparing the gene components and gene organization in the genomes among species is called comparative genomics [TAKAKAZU *et al*., 2002].

The level of 16SrDNA sequence similarity has been proposed as a basis for bacterial species definition [STACKEBRANDT and GOEBEL, 1994] and the use of polyphasic taxonomy has been advocated to ensure well–balanced determinations of taxonomic relationships [VANDAMME *et al*., 1996].

Little genome information is available concerning endophytic bacteria and *Klebsiella* genome studies will drive

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new research into this less–understood, but important category of bacterial–plant host relationships, which could ultimately enhance growth and nutrition of important agricultural crops and development of plant–derived products and biofuels [FOUTS *et al.*, 2008].

Therefore this work was conducted to study the genetic relationship between local *Klebsiella sp.* isolated from groundnut from Gadarif (Sudan) and some American type culture collections.

Material and methods

The local bacterial strain was isolated from root nodules of groundnut grown in Gadarif which is one of the most agricultural region in Sudan.

It is located in the eastern part of Sudan and characterized by heavy clay cracking soils (Vertisols).

For isolation of the bacteria Yeast Extract Mannitol Agar (YEMA) medim was used as described before in [IDRIS *et al.* 2015].

DNA extraction

DNA isolation and amplification were also described before in [IDRIS *et al.* 2015]. Genomic DNA was isolated similarly as described by [DHAESE *et al.*, 1977].

The bacteria was grown in AG (Arabinose Gluconate) broth medium in incubator shaker (150 rev/minutes) at 28°C for 2 days. About 20 mL of the bacterial culture were collected by centrifugation. After washing the bacterial biomass once with TE buffer (10 mM tris, 1 mM EDTA, pH 8), bacteria were resuspended in 300 μL TE buffer. 100 μL of 5 % SDS (Sodium dodecyl sulfate) and 100 μL pronase E (2.5 mg/mL in TE buffer pre–incubated for 90 minutes at 37°C) were added.

After mixing, the solution was incubated for overnight.

Then the DNA was thoroughly sheared using a syringe. The DNA was purified by two extractions with 300 μL of Tris–buffered phenol and one extraction with methylene chloride. DNA was precipitated with 2.5 volumes of ethanol.

The quality and quantity of the DNA were assessed using a NanoDrop ND–1000 device (Spectrophotometer, USA) and agarose gel electrophoresis stained with ethidium bromide, using a marker ladder as reference.

Amplification and sequencing of 16SrRNA gene

For amplification of the *16SrRNA* gene, the forward primer 16Sa (5`–CGCTGGCGGCAGGCTTAACA–3`) and the reverse primer 16Sb (5`–CCAGCCGCAGGTTCCCCT–3`) were used [VAN BERKUM and FUHRMANN, 2000] and amplified near 1500 base pairs of *16SrRNA* from the isolate.

The PCR was performed using 5 μL of 10x *pfu* buffer, 1 μL dNTPs, 1 μL forward primer, 1 μL reverse primer, 1 μL template DNA, 1 μL pfu DNA polymerase, 1.5 μL DMSO (Dimethyl sulfoxide) and 38.5 μL double distilled water total volume of 50 μL.

PCR conditions were: an initial denaturation at 95°C for 3 minutes, 34 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 2 minutes and final extension at 72°C for 15 minutes. PCR products were purified by MEGA quick–spin Total fragment DNA purification kit according to manufacture instructions. For visualization PCR products were separated on 1 % agarose gel and stained with ethidium bromide, using a marker ladder as reference.

The PCR product was sequenced with the above mentioned primers and sequenced again with the primers:
Kleb 16Sf (5–CCCTGGTAGTCCACGCTGTAAACG–3) and
Kleb16Sr (5–TTCGCACCTGAGCGTCAGTCTTTG–3).

Sequencing was done by GATC Biotech (Konstanz, Germany).

The sequences of American type culture *Klebsiella spp.* used in this study were downloaded from Gene Bank data base which were deposited by [DRANCOURT *et al.*, 2001].

Data analysis

The sequence analyses of *16SrRNA* genes were performed by using the algorithm BLASTN [ALTSCHUL *et al.*, 1997], EMBOSS (European Molecular Biology Open Software Suite) was used for merging of DNA sequences and the software programs APE (A Plasmid Editor) was used for the sequences alignments.

Nucleotide sequences accession number

The *16SrRNA* sequence of Gadarif strain was deposited in the Gene Bank data base under the number KJ940119 [IDRIS *et al.* 2015], the downloaded sequences accession numbers were:

The *16SrRNA* sequence of Gadarif strain was deposited in the Gene Bank data base under the number KJ940119 [IDRIS *et al.* 2015], the downloaded sequences accession numbers were: AF129440 for *K. oxytoca* ATCC13182^{T}, AF129441 for *K. ornithinolytica* ATCC 31898^{T}, AF129443 for *K. planticola* ATCC 33531^{T}, AF129444 for *K. trevisanii* ATCC 33558^{T}, AF129442 for *K. terrigena* ATCC 33257^{T} and AF130981 for *K. pneumoniae* subsp. *pneumoniae* ATCC 13883^{T} [DRANCOURT *et al.*, 2001].

Results and discussion

The DNA sequences of the *Klebsiella sp*. isolated from Gadarif (Sudan) and American samples differed at 30 to 31 nucleotide positions except *Klebsiella pneumoniae* was found differed at eight positions only.

The positions of the differences were illustrated in Table 1.

The sequences from the American samples differed from each other in number of nucleotides positions less than that of Gadarif isolate.

All the American culture types differ from Gadarif isolate in position 33 in the same nucleotide, the nucleotide of difference in this position was (C) for the American types and (T) for Gadarif isolate. In position 233 the nucleotide of difference was (G) for Gadarif isolate and it was (A) for all other *Klebsiella spp*. used in this study except for *Klebsiella oxytoca* the nucleotide in this position was (T).

It is also found that all species distinguished from Gadarif isolate in other distinct positions and the same nucleotides.

This observed clearly in positions 417, 809 to 1022, 1139, 1200 and 1201.

However in these positions there were no differences between Gadarif isolate and *K. pneumoniae*.

In the position 458 all species differed from Gadarif isolate except *K. oxytoca*.

On the other hand there were no differences between Gadarif isolate and *K. orinthinolytica, K. planticola, K. trevisanii, K. pneumoniae* and *K. terrigena* in the positions 51 and 86 to 209. In contrast *K. oxytoca* differed totally in its DNA sequences from Gadarif isolate and the other species in the same positions.

The same was true in the positions 1338 and 1350. There were other positions in which *K. orinthinolytica*, *K. planticola*, *K. trevisanii* and *K. terrigena* differed from Gadarif isolate in the same nucleotide, namely the positions 247, 391, 428, 440 and 442.

This relationship may be due to their association with plant as it was previously reported that *K. planticola* and *K. terrigena* are associated primarily with botanical and aquatic environments, whereas *K. ornithinolytica* was first identified from clinical samples [BAGLEY *et al.*, 1981; IZARD *et al.*, 1981; SAKAZAKI *et al.*, 1989].

However in position 440, in addition to the differences between Gadarif isolate and *Klebsiella spp*. mentioned above, also there were differences between our isolate and *K. pneumoniae*.

It is also observed that *K. orinthinolytica*, *K. planticola*, *K. trevisanii* shared the same nucleotide differences in positions 577, 583, 621 and 636 compared to Gadarif isolate.

From the above results it is found that *K.* p*neumoniae* is the most related to Gadarif isolate, because of the less number of nucleotides differences.

The reason of this less differences may be their plant origin, but the differences may be resulted from the different environmental conditions and places from which the isolates were obtained.

So it seems that our isolate is *K. pneumoniae*. At the same time *K. oxytoca* was found the most different from Gadarif isolate, this may be due to its clinical origin [JOAINIG *et al.*, 2010]. Among American types *K. planticola* and *K. trevisanii* were found closely related, they differ only in two positions, 457 and 1120.

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Therefore these species were combined to *K. planticola* [GAVINI *et al.*, 1986] as mentioned before.

On the other hand these latter two species and *K. orinthinolytica* were found differ from each other in six positions, 457, 573, 632, 1118, 1120 and 1123. Thus it was proposed to transfer them to new genus *Raoultella* [DRANCOURT *et al.*, 2001].

Table 1.

Comparison of nucleotide sequences differences of the different *Klebsiella spp*

Positions	Nucleotide						
	Gadarif	*oxytoca*	*Orinthinolytica*	*planticola*	*trevisanii*	*Pneumoniae*	*terrigena*
33	T	C	C	C	C	C	C
51	G	A	–	–	–	–	–
75	C	–	–	–	–	–	T
86	C	T	–	–	–	–	–
120	T	C	–	–	–	–	–
182	T	A	–	–	–	–	–
201	A	T	–	–	–	–	–
209	A	G	–	–	–	–	–
233	G	T	A	A	A	A	A
241	G	A	–	–	–	–	A
247	C	–	T	T	T	–	T
391	G	A	A	A	A	–	A
417	C	T	T	T	T	–	T
428	G	–	A	A	A	–	A
440	G	–	T	T	T	A	T
442	G	–	A	A	A	–	A
456	T	–	–	–	–	C	–
457	A	–	–	–	G	–	–
458	T	–	G	G	G	G	G
472	C	–	T	T	T	–	T
573	C	–	–	T	T	–	–
577	C	–	T	T	T	–	–
583	G	–	A	A	A	–	–
620	T	–	–	–	–	–	C
621	C	–	T	T	T	–	–
632	G	–	–	A	A	–	–
636	A	G	T	T	T	–	–
794	T	–	–	–	–	C	
809	T	C	C	C	C	–	C
821	G	T	T	T	T	–	T
831	C	A	A	A	A	–	A
857	A	G	G	G	G	–	G
970	G	A	A	A	A	–	A
971	G	C	C	C	C	–	C
984	C	G	G	G	G	–	G
992	T	A	A	A	A	–	A
993	C	G	G	G	G	–	G
1003	A	T	T	T	T	–	T
1022	G	C	C	C	C	–	C
1118	G	T	T	–	–	–	–
1119	C	–	–	–	–	T	–
1120	A	–	–	C	–	–	–
1123	C	T	T	–	–	–	–
1139	A	G	G	G	G	–	G
1200	C	G	G	G	G	–	G
1201	C	T	T	T	T	–	T
1291	T	–	–	–	–	–	C
1312	A	–	–	–	–	–	G
1338	A	G	–	–	–	–	G
1139	A	–	–	–	–	G	–
1350	T	C	–	–	–	–	C

The majority of the differences mentioned above were occurred at several distinct positions.

These positions may play role in adaptation of these bacteria to live in different environmental conditions which agrees with the results obtained before that many of the plant–induced genes in *Klebsiella pneumoniae* Kp342 appear to be involved in the adaptation of bacteria to conditions within plant tissue, such as the limitation of amino acid and carbon source concentrations [FOUTS *et al.*, 2008].

Also recently we reported that the differences in the sequence alignment of *Klebsiella spp.* isolated from different regions in Sudan lays in the region between 801 to 1000 bp of the sequences, this may indicate that these isolates are of the same ancestor and changes occur in this region of *16SrRNA* genes because of different environment and different soil types from which the strains were obtained.

Hence the soil type has a direct effect on the genetic characters of the isolates and the changes in this region enable these isolates to adapt themselves to live in the different soil types [IDRIS *et al.*, 2015; VASILEVA and LLIEVA, 2012].

The specification of differences in DNA sequences for *Klebsiella* isolated from different regions helps to detect the reasons of less or inefficiency of some of these bacteria in crop inoculation, especially when isolated and used as inoculant in different region.

So it can be used as tool for recombinant DNA technology to maintain the inefficient strains.

However studies revealed that despite the *16SrRNA* gene is efficient to define genera, because it is conserved, but have variable regions; it has also limitations to identify species, due to possible occurrence of genetic recombination and horizontal gene transfer [NETO *et al.*, 2010].

Moreover, several studies have shown that *16SrRNA* genes may undergo recombination and horizontal transfer resulting in sequence mosaicism [NETO *et al.*, 2010; VAN BERKUM *et al.*, 2003].

Another disadvantage of bacterial identification based on the analysis of *16SrRNA* genes is that, closely related species cannot always be differentiated because of high levels of sequence conservation [VINUESA *et al.*, 2005].

To surmount these difficulties, the use of other genes such as protein coding (housekeeping) genes with greater sequence divergence than *16SrRNA* genes, are recommended [VINUESA *et al.*, 2005; Martens *et al.*, 2007].

Finally more studies are required concerning these strange bacteria, *Klebsiella* which fix nitrogen to plant [SEVILLA *et al.*, 2001] and Human pathogen [FOUTS *et al.*, 2008] at the same time.

Conclusions

The study concluded that, *Klebsiella spp.* isolated from different regions and different environmental conditions differ in their DNA sequences.

The approach of comparing DNA sequences detect the differences through the most related bacterial species and identify mutated positions.

This will help to resolve the less or inefficiency of crops inoculation with some of these bacteria through recombinant DNA technology.

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